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Title of Thesis: EVIDENCE FOR THE EXISTENCE OF A BONE MARROW-
BLOOD BARRIER FOR THE PASSAGE OF SPECIFIC
COMMITTED STEM CELLS IN HUMANS AND CANINES
AND THEIR PHYSICAL SEPARATION FROM
LYMPHOCYTES AND PLURIPOTENT STEM CELLS

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ABSTRACT

Title of Dissertation: Evidence for the Existence of a Bone Marrow-Blood Barrier for the Passage of Specific Committed Stem Cells in Human and Canine and Their Physical Separation From Lymphocytes and Pluripotent Stem Cells

Thomas Jose Contreras, Doctor of Philosophy, 1982

Dissertation directed by: Siegmund J. Baum, Ph.D.
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The spontaneous presence of hematopoietic stem cells in peripheral blood of mammals has long been recognized. Although present in peripheral blood in small numbers, their existence may be of physiological significance in the maintenance of a hematopoietic homeostasis at sites of blood cell formation distributed throughout skeletal cavities and lymphatic tissues. Transplantations of hematopoietic stem cell collected from peripheral blood are known to reconstitute the bone marrow and lymphatic organs of lethally irradiated animals. However, the characterization of these peripheral blood stem cells and the mode of their selection for migration into peripheral blood from the bone marrow remains to be resolved. To date, the murine bone marrow and circulating stem cells have been better characterized than those of larger mammals. Very recently, it has been postulated that in dogs there is a bone marrow-blood barrier capable of selecting a specific granulocyte/macrophage population of stem cells for migration into blood. This investigation utilizes the principle of counterflow centrifugation elutriation (CCE) for the broader enunciation of this theory in the canine and to postulate a similar theory for humans. CCE was also used for the clean physical separation of pluripotent and committed stem cells obtained from the peripheral blood of dogs. Human subjects were leukapheresed for collection of spontaneously circulating stem cells. Dogs were infused with dextran sulfate for the

mobilization of hematopoietic stem cells into peripheral blood and collected for elutriation by the establishment of a leukapheresis procedure for small dogs. The isolation of committed stem cells was monitored by in vitro culture assays and the presence of the pluripotent stem cell was ascertained by the complete hematopoietic reconstitution of lethally irradiated animals. CCE fractionation of human peripheral blood stem cells revealed homogeneous subpopulations of granulocyte/macrophage (CFU-GM) and macrophage (M-CFC) progenitor cells, in lieu of heterogenous populations found in bone marrow, indicative of the existence of a bone marrow-blood barrier in humans capable of stem cell subpopulation selection for migration into peripheral blood, as previously postulated for the canine. CCE fractionation of dog peripheral blood stem cells also resulted in homogeneous circulating subpopulations of CFU-GM and M-CFC as well as CFU-F (stromal cell precursor) and CFU-M (megakaryocytic precursor). These new findings support the hypothesis of a bone marrow-blood barrier in dogs selective not only for CFU-GM subpopulations as contented by others, but also for M-CFC, CFU-F, and CFU-M subpopulations. Fractionation data obtained from dextran sulfate mobilized and non-mobilized animals were analogous, supporting the contention of others, that dextran sulfate does not alter the selectivity of the bone marrow-blood barrier despite enhancing the movement of stem cells across the barrier. Autologous transfusions of CCE fraction I cells, containing less than 4% of all measured committed stem cell activity, were capable of reconstituting the bone marrow of lethally irradiated dogs. Autologous transfusions of CCE fraction III cells containing greater than 95% of all committed stem cell activity measured were not capable of repopulating the bone marrow of lethally irradiated dogs. These original findings are indicative of the separation of committed stem cells from the true hematopoietic pluripotent stem cell by counterflow centrifugation elutriation. In addition, the committed stem cells were

fractionated with low numbers of residual lymphocytes. Comparison of peripheral blood and bone marrow CFU-GM populations, in human and canine, disclosed that the average peripheral blood CFU-GM population was larger than the average bone marrow CFU-GM population.

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by

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DEDICATION

To my Father and Mother for their love and
moral support, who wanted me to achieve this goal
as much as I did.

To my wife Gloria for her ever present
love, support and encouragement.

To my daughter, Naomi, and son, Tommy for
their consideration and understanding.

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INTRODUCTION

The existence of hematopoietic stem cells in the peripheral blood has long been established. Hematopoietic stem cells harvested from peripheral blood have been shown to be able to repopulate the bone marrow and lymphatic tissues of lethally irradiated animals. Although present in blood in comparatively small numbers, their presence may be of physiological importance in the maintenance of hematopoietic homeostasis throughout the various skeletal sites involved in hematopoiesis. The characterization of these stem cells circulating spontaneously and the mode of their selection within the bone marrow for mobilization into peripheral blood remains to be determined.

Recent evidence suggests the existence of a bone marrow-blood barrier permitting only specific subpopulations of granulocyte/macrophage committed stem cells for mobilization into the peripheral blood. The presence of such blood barriers are known in other areas of the body. For instance, the blood-cerebrospinal fluid barrier and the blood-brain barrier, exist between the blood and the cerebrospinal fluid and extracellular brain fluid, respectively. Dextran sulfate has been shown to mobilize larger numbers of stem cells from extravascular sites without altering the bone marrow-blood barrier selectivity.

Characterization of the murine circulating and bone marrow stem cell populations has been rather extensive. However, in humans and in larger animals, like the dog, stem cell characterization has been limited by the inability to identify directly the stem cell essential for complete hematopoietic reconstitution of lethally irradiated animals. Another limitation in the characterization of hematopoietic stem cells is the finite number of techniques available for the isolation and concentration of these cells types without physical alterations or irreversible damage.

With the advent of counterflow centrifugation elutriation and its ability to separate cells with similar physical properties, the further elucidation of the bone marrow-blood barrier hypothesis, and the characterization of peripheral blood stem cells was taken one step forward. Furthermore, the physical separation of the pluripotent and committed stem cells was also accomplished.

A review of the current knowledge of hematopoiesis and its regulation, of stem cell morphology and physical characteristics, as well as that of the bone marrow hematopoietic microenvironment is presented. Background material is presented on the immunological complications of the presence of immunocompetent lymphocytes in stem cell transfusions and the hematopoietic death syndrome. Also presented is a review of the methodology for the mobilization of hematopoietic stem cell into the peripheral blood, the procurement of these stem cells from peripheral blood and a discussion of the principle of counterflow centrifugation elutriation.

BACKGROUND LITERATURE

PHYSICAL CHARACTERISTICS OF STEM CELLS

The morphology of stem cells is incompletely described, though present evidence suggests that it resembles lymphocytes superficially (Yoffey, 1973). Although resembling lymphocytes, stem cells do not possess the immunological characteristics or antigenicity of either T or B lymphocytes and have been categorized as a null cell (Janossy et al., 1980).

In highly purified stem-cell populations, a small mononuclear cell has been identified, by electron microscopy, with certain fine structural characteristics that would be expected of stem cells that are thought to differentiate it from the lymphocyte. Dicke et al. (1973) and van Bekkum et al. (1971) have reported morphological characteristics of a candidate stem cell. These electron microscopic characteristics included a round nucleus with indentations, one or two large nucleoli, finely dispersed or flocculent chromatin and a small brim of cytoplasm filled with predominately free ribosomes. A few cytoplasmic vesicles are present and mitochondria are relatively scarce and small. Organelles present in small lymphocytes but not present in candidate stem cells are golgi apparatus, endoplasmatic reticulum, lysosomes, and multivesicular bodies.

Despite the uncertainties regarding the appearance of stem cells, many of their functional and physical characteristics are known (Metcalf and Moore, 1971; Testa and Lajtha, 1973). Pluripotent stem cells are separable from the committed granulocytic stem cell by kinetic analysis (Metcalf and Moore, 1971). The committed population of cells is active in cell cycle (approximately 30 per cent in DNA synthesis at any given time) whereas most of the pluripotent stem cells are not synthesizing DNA but are in a 'resting' (G_0) or prolonged G_1 phase

(Metcalf and Moore, 1971). Thus, although cell-cycle-specific chemotherapeutic agents may kill an appreciable number of committed granulocytic stem cells, the pluripotent stem cell may not respond to agents that depend on inhibition of DNA synthesis for their activity.

HEMATOPOIESIS

Evidence from recent research suggests the compartmentalization of hematopoiesis which is outlined in Figure 1 (Golde and Cline, 1974; Robinson and Mangalik, 1975). A stem cell may be defined as a cell with the capacity both to replicate itself and to produce more differentiated daughter cells (Boggs and Chervenick, 1970). Therefore, a stem cell population is self sustaining. These hematopoietic precursor cells have different potentialities for maturation or differentiation. The pluripotential stem cell is uncommitted and capable of differentiating along all blood cell lines, i.e., erythrocytes, granulocytes, monocytes-macrophages, and platelets. Evidence for the presence of pluripotent stem cells derives from observations on the formation of mixed colonies of erythroid, granulocytic, monocytic, and megakaryocytic cells that appear in the spleen when appropriate concentrations of murine bone marrow cells are injected into lethally irradiated recipient mice (Metcalf and Moore, 1971; Till and McCulloch, 1961). The spleen colony assay developed by McCulloch et al. (1973) is now used to measure the pluripotent stem cell or CFU-S (colony-forming unit, spleen) in mice.

There is thought to be a more primitive pluripotent stem cell which is capable of forming all the blood elements including the cells of the immunocyte line, i.e., the lymphocytes and plasma cells (Boggs et al., 1970; Metcalf and Moore, 1971; Nowell and Wilson, 1971). Nowell and Wilson (1971) and Wu et al. (1968) have presented evidence for the existence of this cell. This evidence is

FIGURE 1.

Diagrammatic representation of the current concept of hematopoiesis and its humoral control by substances secreted by adult blood cells or cells within the kidney. The hematopoiesis compartmentalization theory includes the pluripotent, committed, differentiated, blood, and tissue compartments. The pluripotent stem cell compartment is composed of the primitive pluripotent stem cell with the capacity to form all the blood elements including the lymphoid line, and the pluripotent stem cell with the ability to differentiate along the erythroid, granulocytic, monocytic, and megakaryocytic cell lines. CFU-S, colony forming unit-spleen; CFU-M, colony forming unit-megakaryocyte; M-CFC, macrophage-colony forming cell; CFU-GM, colony forming unit-granulocyte and macrophage; ERC, erythropoietic-responsive cell compartment; BFU-E, burst forming unit-erythrocyte; CFU-E, colony forming unit, erythrocyte; CSA, colony stimulating activity; NRA, neutrophil releasing activity; EP, erythropoietin; RBC, red blood cell; MACRO, macrophage; LYMPH, lymphocyte; ENDOTH, endothelial cell; GRAN, granulocyte; MONO, monocyte. (From Golde and Cline, N. Eng. J. Med. 291:1388, 1974 and Robinson and Mangalik, Seminars in Hematology 12:7, 1975).

MODEL OF HEMATOPOIESIS

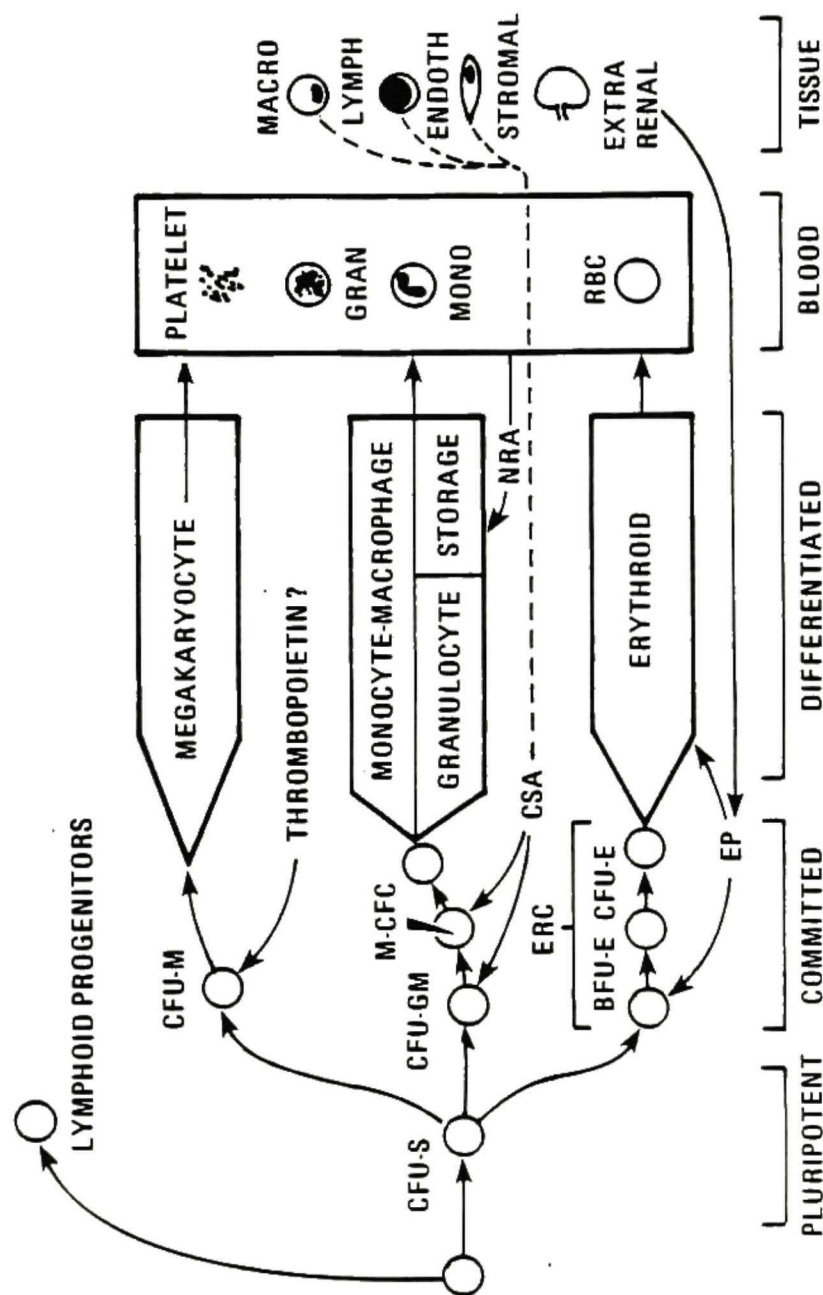


Figure 1

derived from observations of the appearance in recipient mice of spleen and lymphoid tissue colonies containing replicating lymphocytes of donor origin with marker chromosomal abnormalities. The cytogenetic abnormalities were induced by irradiation of the bone marrow donor animal. These studies suggest that a stem cell exists with the capacity for differentiation along both the lymphoid and myeloid cell pathways. Thomas et al. (1972) suggested that the existence of common stem cells for the lymphoid and hematopoietic cell lines is related to the reconstitution of both the hematopoietic and immunologic systems when a successful bone marrow transplantation is performed in man or animals.

The more mature unipotential stem cells are committed to differentiate along one of the three hematopoietic cell lines. The CFU-C or more recently called CFU-GM will differentiate to form colonies of granulocytes and macrophages in in vitro culture assays (Bradley et al. 1969). The BFU-E (burst-forming unit, erythrocyte) and the CFU-E (colony forming unit, erythrocyte) are committed stem cells and will differentiate into erythrocytic colonies and produce red blood cells (Erslev, 1972). Finally, the CFU-M (colony -forming unit, megakaryocyte) will form colonies of megakaryocytes which produce platelets. The adaptation of these in vitro culture techniques for the detection and quantitation of the committed unipotent stem cells are well described in the current literature (Debelak-Fehir, et al., 1975; Faille, et al., 1981; Hansen et al., 1980; van Bekkum and Dicke, 1972). These techniques provide a useful tool for prediction of the likelihood of bone marrow engraftment, as well as, monitoring the efficiency of concentration and isolation of the progenitor cells (Dicke and van Bekkum, 1972). The in vitro cloning technique has also proven to be an extremely valuable tool in the elucidation of the humoral control of hematopoiesis.

REGULATION OF HEMATOPOIESIS

The most important hematopoietic regulatory factors appear to operate at the stem cell level. Thus, erythropoietin, the hormone concerned with erythropoiesis, acts on a committed or unipotent stem cell CFU-E (Krantz and Jacobson, 1970). This cell is the target for the hormone, which is believed to interact with a receptor on the cell surface, thereby triggering cell division and the differentiation along the erythrocytic pathway (Chang et al., 1974; Erslev, 1972; Krantz and Jacobson, 1970). The committed stem cells of the megakaryocytic (CFU-M) and granulocytic (CFU-GM) cell lines are thought to respond to thrombopoietins (Shreiner and Levin, 1973) and granulopoietins-CSA (colony stimulating activity) (Stohlman et al., 1973) in a similar fashion.

Hematopoietic stem cells are conceptually described as being compartmentalized or pooled within the bone marrow (Figure 1). This stem-cell hypothesis regarding pools of committed progenitor cells raises the theoretical question of 'stem-cell competition'. This concept postulates that an increased demand for mature cells of one cell line can result in a depletion of committed stem cells of another cell line that may then be reflected in decreased production of mature cells of that other cell line (Stohlman et al., 1973; Hellman and Grate, 1967). Thus, if the pluripotent stem-cell compartment is restricted in size, an increase demand for erythrocytes could lead to neutropenia or vice versa. Although this type of competition occurs in the in vitro state (Hellman and Grate 1967; Lawrence and Craddock, 1968; Metcalf, 1968; Stohlman et al., 1973), the clinical implications of this phenomenon are unknown.

Although considerable attention has been focused on the interaction of circulating hormones with committed stem cells, very little is known about the factors that regulate cellular differentiation from the pluripotent to the

committed or unipotent stem-cell compartment (Boggs and Chervenick, 1970; McCulloch et al., 1973; Metcalf and Moore, 1971). A current hypothesis states that pluripotent stem cells "sense" the utilization of the committed stem cells and that this signals or induces a feed-in from the pluripotent to the committed stem-cell pool (Erslev, 1972; Lajtha et al., 1962). According to this theory, a granulopoietin acting on the target-committed precursor cell causes depletion of the cells in that compartment, thereby initiating a recruitment of more granulocyte-committed cells from the pluripotent stem-cell compartment. The 'sensing mechanism' is believed to involve short-range cellular interactions (McCulloch et al., 1973; Metcalf and Moore, 1971).

THE BONE MARROW HEMATOPOIETIC MICROENVIRONMENT

The "sensing mechanism" purposed in short-range cellular interaction of the pluripotent hematopoietic stem cell may involve interactions with local or microenvironmental factors within the hematopoietic organs (Lajtha, 1979). Weiss (1975) and Lichtman (1981) have defined the hematopoietic microenvironment as those elements within the hematopoietic tissue which provide mechanical support for the developing hematopoietic cells, as well as to other non-hematopoietic elements, including the vascular and neural tissue of the bone marrow. The functional concept of the hematopoietic microenvironment can broadly apply to the totality of factors which influence hematopoietic activity. Bentley (1982), however, in discussing the function of the hematopoietic microenvironment states, "the term is more usually applied to factors which act at close range rather than at a distance and which are essentially permissive rather than regulatory. Perhaps the most compelling evidence supporting the existence of a permissive hematopoietic microenvironment derives from the localization of hematopoiesis to specific sites within the body."

Although, it has been well established by bone marrow engraftment studies that pluripotential stem cells circulate freely in the peripheral blood (Everett and Perkins, 1976), it is only in the hematopoietic tissue that hematopoietic stem cells proliferate and differentiate and not elsewhere (Till and McCulloch, 1961). These observations strongly suggest that certain organs provide microenvironments which are unique in their capacity to support hematopoiesis. How pluripotent or unipotential stem cells escape these organs to circulate freely is not understood.

The bone marrow tissue, which provides the hematopoietic microenvironment, is comprised of cells collectively called stromal cells. The stromal cell population, on the other hand, is comprised of fibroblasts, endothelial cells, fat cells and "reticular cells" (Weiss, 1975). The characteristics and the regulatory role of each of these cell types are unknown. Studies (Dexter et al., 1977; Reimann and Burger, 1979) have demonstrated that murine marrow derived adherent cells (MDAC) consisting of fibroblasts, macrophages, epithelioid cells, and fat cells, have the capacity to sustain the proliferation of pluripotent stem cells for prolonged periods in vitro. Bentley and Foidart (1980) observed that the vast majority of MDAC produce collagen fibers confirming their contention that these cells were fibroblasts. A liquid culture system has been used to clone and to characterize human, dog, and murine fibroblast colony-forming cells (CFU-F) (Castro-Malaspina et al., 1978; Wathen et al., 1981; Werts et al., 1980a; Wilson et al., 1978). The CFU-F activity is becoming a useful indicator of the presence of a hematopoietic microenvironment and its capability to support hematopoiesis. We can only speculate on the extent of the involvement of the hematopoietic microenvironment in a hypothetical bone marrow-blood barrier.

IMMUNOLOGICAL COMPLICATIONS OF THE PRESENCE OF THE IMMUNE COMPETENT CELL, THE LYMPHOCYTE, IN LEUKAPHERESIS CONCENTRATES

Transfusions of stem cells obtained from peripheral blood containing lymphocytes, have been implicated in graft-versus-host disease (GVHD) in allogeneic transplantations (Storb et al., 1967, 1969, 1970, 1973). Removal of immunocompetent cells from bone marrow in vitro prior to transplantation does not prejudice engraftment but does prevent early GVHD in unrelated recipients (Dicke and van Bekkum, 1971). Storb et al. (1970, 1973) have implicated the T-lymphocyte as the mononuclear cell responsible for GVHD. Thus, the use of peripheral blood as a source of stem cell procurement, appears to go against all immunological doctrine, since T cells are more abundant in peripheral blood than in bone marrow. However, engraftment of transplanted bone marrow is apparently enhanced by the additional transfusion of peripheral blood cells (Storb et al., 1977). It has been proposed that this effect may reflect the stimulation of hematopoietic progenitor cells in the bone marrow by T-lymphocytes in the blood (Barr et al., 1977), a contention for which experimental support has since been provided in dogs by Deeg et al. (1979). Such benefit may derive from the selective influence of T-lymphocyte subpopulations in human hematopoiesis (Barr and Stevens, 1982); "helper" cells predominate in the peripheral blood and "suppressor" cells predominate in the bone marrow (Janossy et al., 1980).

Despite the enhancement of engraftment by the "helper" T-lymphocyte, the "killer" T-lymphocyte still manifests itself and consequently, the incidence of GVHD in allogeneic transplantations remains a serious problem. For this reason, attempts to reduce or eliminate the number of lymphocytes from leukapheresis concentrates (Korbling, et al., 1977; Ross et al. , 1977) and bone marrow (Dicke and van Bekkum, 1970, 1971, and 1972) have been undertaken. These studies

utilize gradient density centrifugation techniques which are open systems and difficult to maintain aseptic conditions. The procedures are also cumbersome and time consuming. Counterflow centrifugation elutriation may provide a clean, aseptic procedure for the separation of stem cells from lymphocytes.

HEMATOPOIETIC DEATH SYNDROME

To date, no assay has been developed for the detection and quantitation of the pluripotential stem cell in any species above the murine. Fauser and Messner (1978 and 1979) have described an in vitro culture for a human pluripotent stem cell measured by growth in single colonies of granulocytic, erythrocytic, monocytic, and megakaryocytic cell types (CFU-GEMM). The CFU-GEMM possess at least some features analogous to the pluripotent murine splenic colony-forming unit (CFU-S). However, no culture assay for the CFU-GEMM has been described for the dog. Since the presence of the pluripotent stem cell equivalent to the murine CFU-S is essential for complete hematopoietic reconstitution of the bone marrow of all species, the only viable method for directly measuring the presence of the pluripotent stem cell in dogs is via the in vivo approach. One such approach is by the complete bone marrow reconstitution of lethally irradiated beagles.

Processes of radiation-induced lethality in mammals can be conveniently divided into three distinct biological events, which may be separated temporally into the hematologic syndrome (HS), the gastrointestinal syndrome (GIS), and central nervous system syndrome (CNSS). The 'hematopoietic syndrome' has the lowest-dose mortality-experience and is the last to occur in time. Mortality usually occurs before four weeks for small animals or before 6-8 weeks for larger animals (Jones, 1981). Different species, having different survival times to the same irradiation dose, reflect a 'mortality incidence', i.e., the capacity to survive a given dose. For example, the mean dose lethal to 50% of the population of

beagles, dying at 30 days after exposure to low linear energy transfer (low-LET) radiation ($LD_{50(30)}$), is commonly found to be about 2.5 Gy. Hansen et al. (1961) reported the $LD_{50(30)}$ of beagles exposed to X-irradiation as 2.4 Gy (midline tissue dose) and Norris et al. (1968) reported the $LD_{50(30)}$ of beagles exposed to ^{60}Co gamma-irradiation to be 2.58 Gy. In certain mice the $LD_{50(30)}$ is found to exceed 700 rads (7.0 Gy), but in man no individual is known to have survived a single whole-body uniform exposure to 5.7 Gy, as reported by the National Council on Radiation Protection and Measurements (1979).

Generally, leukocyte and platelet depression is responsible for hematologic failure (Andrews and Haywood, 1971; Robinson, 1968), which induce death by infection and hemorrhage. Recovery depends, primarily, upon maintenance of a critical level of neutrophils and platelets in the peripheral blood (Bond and Robinson, 1967).

The cytotoxic response of mammalian cells irradiated in vitro (cultures) is remarkably constant (except for phase of the cell division cycle; Alper, 1975) for different species and different tissues (Okada, 1970; Puck et al., 1957). The response of cells in vivo often depends upon the rate of proliferation (Bond et al., 1965) and marrow stem cell response seems to vary much less markedly, than would be indicated by strain and species variation of LD_{50} (Gould and Clifton, 1979; Werts et al., 1980b).

There are several features that may account for the variability in mammalian lethality. Bond and Robinson (1967) have reported that the ability of organisms to survive irradiation exposures vary greatly in the minimum levels of stem-cell viability. For example, mice can survive 0.2% minimum level of stem-cell activity, while dogs can not survive lower than 7-11% minimum levels of stem cell activity. Hematopoietic progenitor cells, having different rates of proliferative activity, reflect different radiosensitivities (Alper, 1975).

Furthermore different species have varied time intervals from cell division to mature differentiated cells in the blood.

The second and third, according to dose, mortality events, but ranking second and first in the time of onset, respectively, are the GI and CNS syndromes. These syndromes occur at successively higher dosages and result from gastrointestinal and central nervous system damage. The gut progenitor cells are unipotent, while the nerve tissue is highly differentiated and nonrenewing. The survival time of the animal can usually be an indicator of the primary mode of death, with hematologic death being about three times later than GIS death, which in turn is about three times later than CNSS death (Andrews and Haywood, 1971). Typical figures for mice are hours for CNSS, up to four days for GIS, 5-8 days for bacteremia or mixed GIS and HS death, and 9-30 days for hematopoietic death (Puro and Clark, 1972).

Jones (1981) has, by normalizing doses to fraction of LD_{50} (D/LD_{50}) on a variety of mammalian species, formulated that for uniform dose profiles within any species, D/LD_{50} s of less than 0.54 always results in 100% survival, while D/LD_{50} s greater than 1.3 results in 100% mortality. The calculated LD_{100} (dose resulting in 100% mortality) for beagles, using the reported LD_{50} s of approximately 2.5 Gy (Hansen et al., 1961; Norris et al., 1968), is 3.25 Gy. Norris et al, (1968) reported an LD_{100} for beagles of 3.5 Gy, being within reasonable agreement with Jones formula, but George et al. (1968) and Pitchford and Thorp (1968) reported LD_{100} s of 2.85 Gy for beagles. These LD_{100} s doses are far below the 6.0 Gy dose used in the following studies.

PERIPHERAL BLOOD STEM CELL PROCUREMENT

Investigations in humans and canines have shown that cells capable of repopulating the hematopoietic system are present in small numbers in the

peripheral blood (Cavins et al., 1964; Debelak-Fehir et al., 1975; Epstein et al., 1966; Flidner et al., 1976; Levin et al., 1963; McCredie et al., 1970, 1971; Ross et al., 1975, 1976). The peripheral blood, thus, represents a potential source of pluripotential and unipotential progenitor cells for marrow grafting purposes and characterization studies. The advantages in the procurement of these stem cells from peripheral blood in lieu of bone marrow are numerous. Patients requiring autologous bone marrow engraftments may be poor risks for general anesthesia, especially if multiple transplants are required (Deisseroth et al., 1980). Prospective donors may occasionally refuse to donate bone marrow but will instead contribute peripheral blood (Rich et al., 1980). This may be the case especially when an unrelated donor may be used with increasing frequency (Hansen et al., 1980). Furthermore, donors of peripheral blood stem cells could also provide red blood cells, granulocytes, or platelets by appropriate modification in the collection procedure.

Because of the small number of stem cells in peripheral blood, it has not been possible to obtain large enough quantities of concentrated stem cells from the peripheral blood for clinical use or for detailed studies of the cells' morphology, function and population characterization without use of more sophisticated means than direct blood collections. Leukapheresis for the concentration of larger numbers of peripheral blood stem cells of human and canine has been well documented (Flidner et al., 1976; Kovacs et al., 1978; Lasky et al., 1982; Nguyen and Perkins, 1979; Ross et al., 1975). The leukapheresis procedure entails the selective collection of large numbers of circulating mononuclear leukocytes in a time span of four to five hours. As illustrated in Figure 2, blood from the donor is removed from one leg (arm in humans) and passed through the IBM 2997 Blood Cell Separator where the cell type desired is

FIGURE 2.

Schematic presentation of the continuous flow centrifugation leukapheresis (CFCL) of small beagles on the IBM 2997 Blood Cell Separator for the collection of peripheral blood leukocytes and hematopoietic stem cells using the single stage blood separation channel. The dual stage blood separation channel was used in the collection of platelets for support of lethally irradiated dogs.

CONTINUOUS FLOW CENTRIFUGATION LEUKAPHERESIS OF DOGS ON THE IBM 2997 BLOOD CELL SEPARATOR

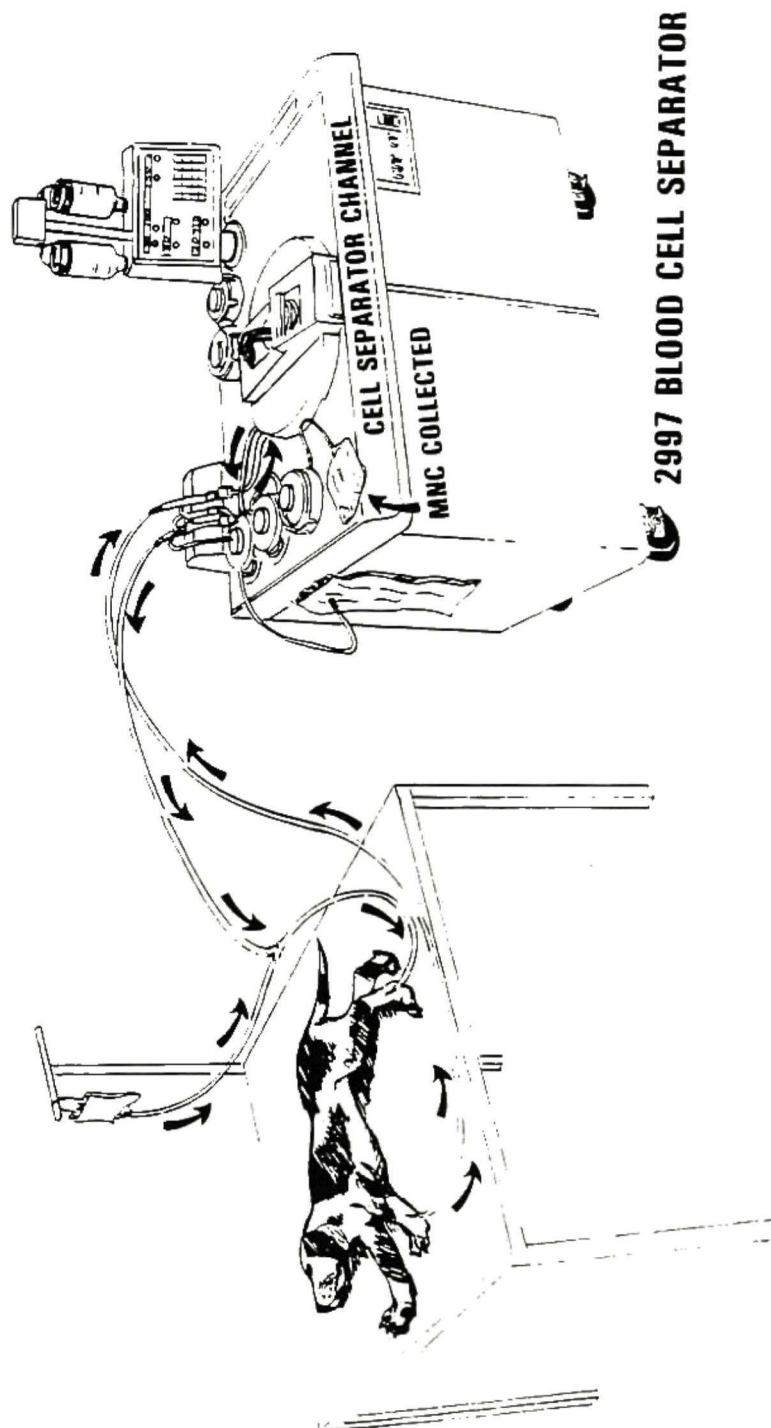


Figure 2

selected and removed. The selected cell type is then collected continuously and stored in a separate container. All other cell types and plasma are returned to the donor via another leg (or arm). Removal of blood from the donor can proceed either continuously or intermittently depending on the instrument being used.

First generation pheresis instruments utilized the principles of continuous flow centrifugation leukapheresis (CFCL), as did the Aminco and IBM celltrifuge (Graw et al., 1971; McCredie and Freireich, 1971), discontinuous or intermittent flow centrifugation leukapheresis (DFCL or IFCL) as utilized by the Haemonetics Blood Separator (Huestis et al., 1975; Szymanski and Kliman 1973), or continuous flow filtration leukapheresis (CFFL) a principle involving adhesion of granulocytes to nylon filters (Djerassi et al., 1979; Herzig et al., 1972). These instruments were initially used to collect great numbers of granulocytes or platelets. However, with the importance of peripheral blood stem cell isolation, these instruments, except for instruments utilizing the principle of filtration leukapheresis, are now also being used for the collection of large numbers of mononuclear cells with modifications in collection procedures.

The newer, second generation, pheresis instruments, which have just recently become available for clinical and investigative purposes, utilize the continuous flow centrifugation leukapheresis principle. The new instruments are the Fenwal CS 3000 Blood Cell Separator (Bucholz et al., 1979; Lasky et al., 1982; Lin et al., 1980) and the International Business Machines (IBM) 2997 Blood Cell Separator (Hester et al., 1979; Kalmin and Grindon, 1981; McLeod and Sassetti, 1981). These programmable computerized instruments are the state of the art. Again, the initial usage of these instruments have been for the collection of granulocytes and platelets, but our laboratory and others have been modifying their procedures for the collection of mononuclear cells. As described in the Materials and Methods, our laboratory has been successful in the modification of

the IBM 2997 Blood Cell Separator for the collection of large numbers of mononuclear cells from small dogs. The new pheresis instruments have the advantage over the older pheresis instruments in that they require much smaller extracorporeal volumes and thus smaller animals can be leukapheresed without hazard of ensanguination.

These leukapheresis procedures allow for the processing of three to six times the donors' total blood volume with an eventual loss of less than ten percent of the donors' erythrocytes. Approximately 250-450 ml of blood volume is normally collected with normal blood volume maintained by continual re-infusion of fluids during the procedure.

MOBILIZATION OF BONE MARROW CELLS

Despite the fact that leukapheresis procedures enable us to harvest large numbers ($1 - 12 \times 10^9$) of mononuclear cells from blood (Kovacs et al., 1978; Ross et al., 1977), one is not assured of collecting sufficient stem cells for the complete hematopoietic reconstitution of the bone marrow and for population characterization studies. Nothdurft et al. (1977) have reported that between 0.02 and 1.38×10^5 CFU-GM per kg of body weight are required for a successful hematopoietic reconstitution of lethally irradiated dogs. Therefore, there is a need for increasing the number of circulating stem cells prior to leukapheresis.

Various chemical agents have been reported to have the capability to mobilize cells, normally found within the bone marrow, into the peripheral blood and thus, increase the circulating populations of these bone marrow cell types. Some agents specifically mobilize granulocytes. Lowenthal and Parks (1975) have used dextran, while Huestis et al., (1975), McCredie et al., (1974), Mishler et al., (1974), Strauss et al., (1979) have all used hydroxyethyl starch (HES) for increasing granulocyte collections in human donors. Others have used prednisone (Bearden et

al., 1977) and dexamethasone (Higby et al., 1975).

Other chemical agents were found with specific capabilities of mobilizing mononuclear cells, and with them the hematopoietic progenitor cells. Pyran co-polymers and related agents have been used by various investigators (Patchen and Lotzova, 1980; van der Ham et al., 1977; Zander et al., 1980) resulting in the redistribution of the bone marrow. These materials have been shown to exhibit toxic effects (Breslow et al., 1973). Dextran sulphate has been quite successful in the mobilization of CFU-GM, the granulocyte-macrophage precursor cell, in dogs, an order of magnitude over normal circulating levels (Ross et al., 1976 and 1978; van der Ham et al., 1977) with toxic effects observed only with the use of the larger molecular weights materials (Rickett et al., 1953; Walton, 1954). In humans, increases in circulating CFU-GM levels have been reported with exercise (Barrett et al., 1978), ACTH (Barrett et al., 1978), prednisolone (Netzel et al., 1974), hydrocortisone (Morra et al., 1981), endotoxin (Cline and Golde, 1977), and plasmagel (Korbling et al., 1980). Leukapheresis concentrates obtained after chemical agent mobilization of the bone marrow cells have resulted in collection of significantly greater quantities of hematopoietic progenitor cells as reported by Fliedner et al., (1976), Korbling et al., (1980), Nguyen and Perkins, (1979), Ross et al. (1978).

COUNTERFLOW CENTRIFUGATION ELUTRIATION

With the advent of counterflow centrifugation elutriation (CCE) (Contreras et al., 1978a; Lionetti et al., 1977; Sanderson et al., 1976) and modifications of published procedures by Contreras et al., (1978b, 1979, and 1980) and Jemionek et al., (1978, 1979, and 1981) large quantities (1.3×10^9) of highly purified granulocytes were isolated from whole blood and leukapheresis concentrates. The CCE procedure has also been used for the isolation and

purification of large numbers of human lymphocytes and monocytes from leukapheresis concentrates (Contreras et al., 1980; Stevenson et al., 1981; Weiner et al., 1981).

Figure 3 illustrates the principle of counterflow centrifugation elutriation. As shown, the centrifugal force created by the rapid rotation of the separation chamber is countered by the fluid entry at the bottom of the chamber. Since the centrifugal force is maintained constant, lighter (more buoyant) cells exit at the top of the chamber. By slowly increasing the rate of fluid entry, heavier (less buoyant) cells, are forced to exit the chamber. This procedure utilizes various physical properties of the cell (density, shape, volume) to magnify cell differences for possible separation (Sanderson, et al., 1976).

Our earlier work in the isolation of larger numbers of lymphocytes and monocytes from leukapheresis concentrates (Contreras et al., 1980) indicated that the CCE technique was also capable of separating committed stem cells from the immune competent lymphocytes. Previously published stem cell purification studies reported that the pluripotential stem cell was isolated in the same fractions with the committed stem cell (Dicke and van Bekkum, 1970 and 1971; Flidner et al., 1976; McCredie et al., 1970; Ross et al., 1975; Storb et al., 1967). This evidence was important because, if it held true, it meant that a technique now existed with the potential to separate both the pluripotent and unipotent stem cells from the lymphocytes and this would permit the use of allogeneic transplantations with hopes of reducing markedly early GVHD incidences. If on the other hand, only the committed (unipotent) stem cells and not the pluripotent stem cells are separated from the lymphocyte then significant physical characteristic differences must exist between these stem cell populations to result in their physical separation by the CCE technique. Of greater interest was the potential of CCE to isolate and concentrate populations of peripheral blood

FIGURE 3.

Schematic representation of the principle of counterflow centrifugation elutriation (CCE) using the Beckman JE-6 elutriator separation chamber. Although the chamber was rotating at a rapid pace to create a centrifugal force, it was counteracted by flow rate forces at the bottom of the chamber. All cells experienced a 1 x g force.

BECKMAN JE6 ELUTRIATOR SEPARATION CHAMBER

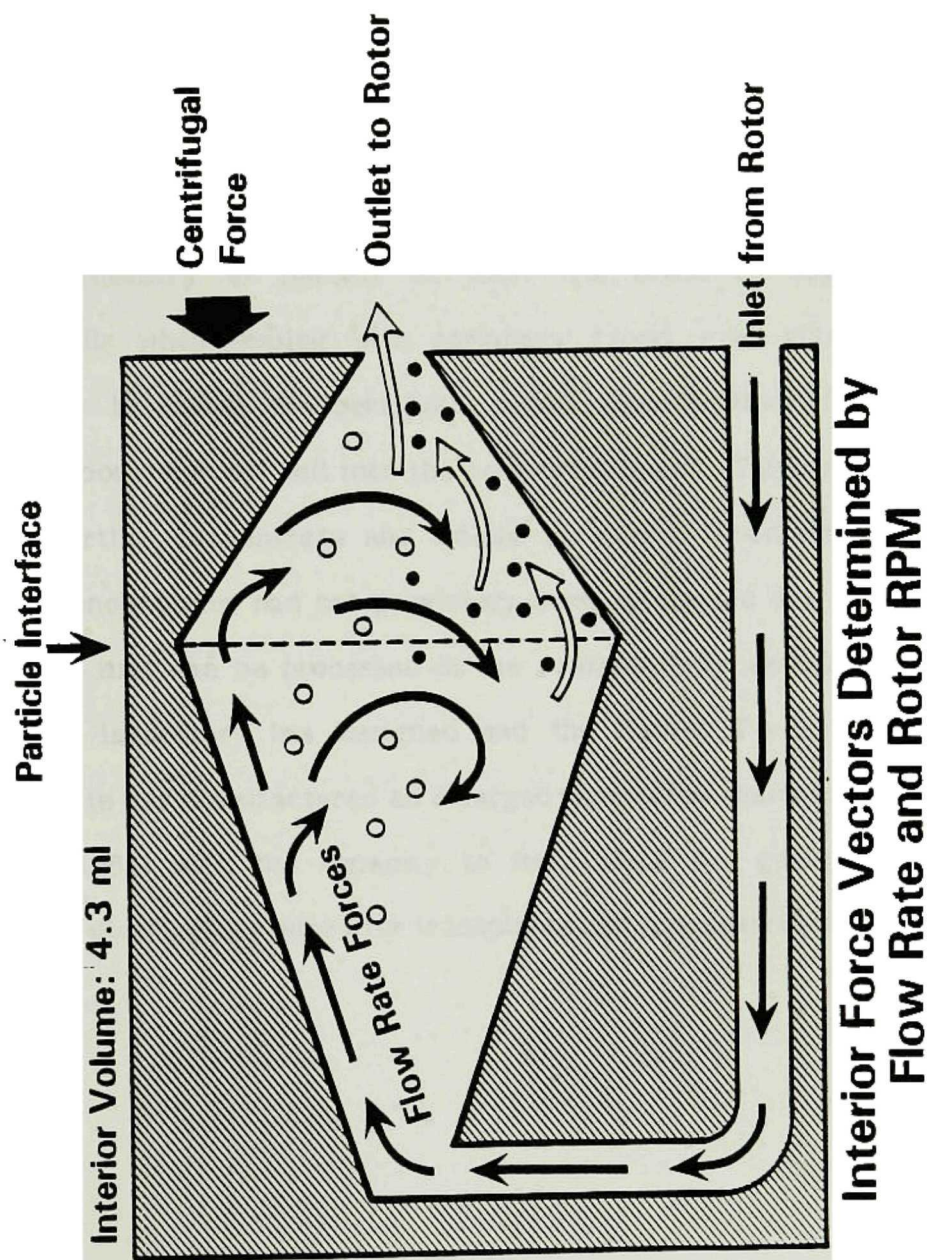


Figure 3

stem cells for their possible characterization. These characterizations could then be compared with CCE fractionated bone marrow stem cell data previously obtained in our laboratory (Jemionek et al., 1982a and 1982b).

Because the concentration of CFU-GM in peripheral blood (per unit number of mononuclear cells), as well as all other stem cells, is lower than that found within the bone marrow by one or two orders of magnitude (Verma et al., 1980), it is necessary to process at least one order of magnitude more mononuclear cells when dealing with peripheral blood, even after the use of chemical agents to enrich the peripheral blood concentration of CFU-GM by mobilization of bone marrow cell into the peripheral blood. Thus, use of the CCE technique to further concentrate and isolate peripheral blood stem cells from leukapheresis concentrates had not previously been attempted due to the limited number of cells that can be processed in the commercially available separation chamber. Our laboratory has designed and the Armed Forces Radiobiology Research Institute has manufactured an enlarged separation chamber (Jemionek et al., 1980 and 1981) with the capacity to fractionate the greater number of peripheral blood stem cell required for transplantation characterization studies.

PURPOSE OF INVESTIGATION

In recent years, considerable evidence has been reported indicating that physical and cytokinetic differences exist between pluripotent and committed stem cells found in the bone marrow and peripheral blood. Hellman and Grate (1968) distinguished two populations of stem cells of mice. One population was rapidly mobilizable from marrow to blood, the other relatively sessile in the bone marrow. Micklem et al. (1975) reported that murine peripheral blood stem cells have less proliferative capacity than those from bone marrow. Giladi et al. (1974) have also reported that bone marrow stem cells are more immature than those in peripheral blood of mice. Verma et al. (1980) performed parallel culture studies on human peripheral blood and marrow cells and confirmed previous observations of Chervenick and Boggs (1971) describing eosinophilic progenitor cell (EO-CFC) colonies of greater frequency in peripheral blood cultures than in those of bone marrow which produced colonies with greater numbers of CFU-GM. These results suggested that the incidence of subpopulations of CFU-GM based on colony morphology were different in marrow and peripheral blood. McCarthy and MacVittie (1978) have shown in mice velocity sedimentation differences in M-CFC population obtained from blood and bone marrow. They reported blood M-CFC velocity sedimentation profiles as being significantly more homogeneous than that of bone marrow. Recently, Gerhartz and Flidner (1980) have shown, in beagles, that blood CFU-GM were smaller than those of the bone marrow and had a lower proportion in S-phase of cell division, as reflected by velocity sedimentation and tritiated thymidine induced cell death rates. Gerhartz et al. (1982) reported that depression of circulating CFU-GM by low-dose whole body irradiation of beagles was associated with a marked shift in the size distribution of granulopoietic progenitor in the bone marrow. They concluded that circulating CFU-GM are not

a random proportion of the bone marrow but a subpopulation of cells which are smaller in size. In an attempt to explain their findings, Gerhartz and Fliedner have postulated the existence of a bone marrow-blood barrier in the dog capable of permitting a select homogeneous population of CFU-GM for passage into the peripheral blood.

The purpose of this investigation was to test the hypothesis that the bone marrow-blood barrier permits only specific stem cells to enter the circulation of humans and canines. Therefore, research was designed to examine, characterize, and measure committed stem cells in the peripheral circulation in order to compare them with profiles of committed stem cells previously obtained from bone marrow aspirates in our laboratory (Jemionek et al., 1982a and 1982b).

An additional aim of this investigation was to attempt to separate physically peripheral blood stem cells from the immunocompetent lymphocytes for possible allogeneic transplantations.

However, physical differences have also been reported between the pluripotent stem cell and the committed granulocyte/macrophage stem cell (CFU-GM). Worton et al. (1969) have shown size differences between CFU-S and CFU-GM in the mouse bone marrow. Haskill et al. (1970) have reported density distribution differences in murine bone marrow CFU-S and CFU-GM. Sutherland et al. (1971) have demonstrated that the CFU-GM harvested from mouse bone marrow suspension cultures were derived from a population of cells which sedimented more slowly than the vast majority of CFU-GM, but had similar sedimentation velocity to pluripotent stem cells (CFU-S). Iscove et al. (1972) reported similar populations of cells with similar sedimentation velocities in normal human marrow. Visser et al. (1980) demonstrated, using a light activated cell sorter, that the CFU-S had a smaller cell diameter than the CFU-GM. Very recently, Jemionek et al. (1982b) have reported physical differences between

pluripotent and committed stem cells of dog bone marrow from evidence obtained from the autologous transfusions of CCE fractions into lethally irradiated beagles. These results have provided evidence that those hematopoietic progenitor cells that form colonies in culture (CFU-GM) are not identical with the pluripotent stem cells. Despite this evidence, to date, the populations of stem cells have not yet been separated in a definitive manner. Therefore, the question arises whether the physical differences between lymphocytes and all stem cells are greater than the physical differences between committed and pluripotent stem cells. This investigation was undertaken, in part, to answer this very question.

Consequently, the primary objectives of this research were to: (1) characterize the populations of spontaneously circulating CFU-GM and M-CFC found in the peripheral blood of humans, in respect to homogeneity in cell volume and cell density, (2) characterize the populations of CFU-GM and M-CFC spontaneously circulating in dogs and those populations whose numbers are greatly increased by dextran sulfate mobilization, (3) characterize, in addition, the dextran sulfate mobilized populations of CFU-F, CFU-M and if possible of BFU-E and CFU-E in dogs, (4) determine the mobilization and separation characteristics of the pluripotent stem cell in dogs by the complete hematopoietic reconstitution of the bone marrow of lethally irradiated dogs. A secondary objective was to determine the engraftment efficiencies, using peripheral blood stem cell, in dogs irradiated with 6.0 Gy and 9.0 Gy gamma radiation. These studies were to be done to determine the limitations of our peripheral blood stem cell procurement capabilities.

In order to accomplish the above objectives, it was essential to establish the methodology to enable us to procure large numbers of peripheral blood stem cells for their possible separation and concentration by counterflow centrifugation elutriation. The methodology involved the following: (1) development of an

efficient procedure to mobilize stem cells from the bone marrow into the peripheral blood of dogs with dextran sulfate, a commercially available heparin analogue, (2) to develop and maximize a leukapheresis procedure for small beagles for the collection of mononuclear cells and peripheral blood stem cells, (3) and the establishment of techniques for counterflow centrifugation elutriation separations chambers to separate physically and concentrate circulating stem cell populations.

MATERIALS AND METHODS

ANIMALS

Thirteen one to two year old male and female beagles, weighing between 8 and 13 kg, were used for irradiation/transfusion studies. Five male beagles weighing 13-18 kg were used as platelet donors. Eight male English-American foxhounds (20-25 kg) were used for the non-mobilized dog leukapheresis collections. All dogs were dewormed and immunized against distemper, rabies, hepatitis, leptospirosis, and parvovirus, at least six months prior to experimentation. The beagles were under veterinarian care for at least two weeks prior to experimentation. Hematological status was monitored during those two weeks. Beagles were housed individually in stainless steel cages during this period and fed a dry food (Purina Dog Chow, Ralston Purina Co., St. Louis, MO.). Tap water was provided ad libitum. Thirty-six male and female beagles (8-13 kg) were used for leukapheresis and dextran sulfate mobilization studies.

All research was conducted according to the principles enunciated in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animal Resources, National Research Council.

DEXTRAN SULFATE MOBILIZATION

Dextran sulfate, molecular weight 8,000 (Sigma Chem. Corp., St. Louis, MO), was dissolved in sterile normal saline to a concentration of 15 mg/ml. Beagles were then administered intravenous bolus dosages of 7.5, 15, and 30 mg/kg body weight. Seven ml blood samples were drawn in EDTA just prior to dextran sulfate injection and at 1, 2, 3, and 5 hrs after administration. Samples were then assayed for CFU-GM content, erythrocyte and leukocyte counts and blood smears prepared for differential examination.

The administration of dextran sulfate on two separate occasions produced greater mobilization, as evidenced by higher CFU-GM activity in the leukapheresis concentrates. The greater mobilization was achieved by administering 15 mg dextran sulfate per kg body weight four days prior to and again one hour prior to leukapheresis.

CONTINUOUS FLOW CENTRIFUGATION LEUKAPHERESIS

After obtaining written consent, leukocytes were taken from normal human volunteers as CFCL concentrates in 300-ml transfer packs in volumes of approximately 275-300 ml by the method described by Graw et al. (1971) and Herzig et al. (1977). All donors were leukapheresed at the Experimental Hematology Section, Pediatric Oncology Branch, National Cancer Institute, NIH, Bethesda, Maryland. Copies of the consent forms requiring donor's signature are reproduced on Appendices 1 and 2. Forms specify the criteria used to select the donors.

Aminco Celltrifuge

The male foxhounds used as donors of mononuclear cell (MNC) were prepared by insertion of an indwelling arteriovenous shunt 24 hours prior to the leukapheresis procedure as previously described by Buckner, et al. (1968) and Shoji and Vogler, (1974). MNC collections were performed on the Aminco Celltrifuge Cell Separator (Fenwal Division, Silver Spring, MD) using the reusable bowl, and leukapheresing the animals for approximately four hours. Approximately 260 ml of leukocyte-rich ACD anticoagulated concentrates were obtained. The concentrates were kept at room temperature and assayed within one hour of collection.

IBM 2997 Blood Cell Separator

Male and female beagles were leukapheresed on the IBM 2997 Blood Cell Separator (IBM Biomedical Systems, Princeton, NJ) as depicted on Figure 2. Beagles were anesthetized with 4 g% (w/v) sodium thiamylal (Bio-Ceutic Labs., Inc., St. Louis, MO) given to effect (~15 mg/kg body weight) prior to leukapheresis. A tracheal tube was inserted into the dog to maintain an open airway and as a precautionary measure. A 19-gauge butterfly needle, with a six-inch tubing extension (Intravenous Injection Set, Sherwood Med. Ind., Inc., St. Louis, MO), was inserted into the cephalic vein and was used as the donor site. A second 19-gauge butterfly needle was inserted into the lateral saphenous vein and became the recipient site. A slow i.v. drip (20 drops/min) was placed on the recipient line to maintain the site open. Heparin (1000 USP units/ml, porcine mucosal, Lympho-Med, Inc., Chicago, IL) was given intravenously to a concentration of 280 units/kg body weight for systemic anticoagulation prior to drawing the blood into the single stage blood separation channel. Five minutes after heparin infusion, the plasma pump on the leukapheresis instrument began to draw anticoagulated blood from the dog. The prime saline bag/bottle contained 2 units/ml of heparin. The anticoagulant bag or bottle contained 40 USP, units/ml of heparin. Lubrication bag or bottle contained only normal saline. The collection bag contained one ml of heparin prior to collection. The anticoagulant pump rate was set at 8.5-9.1 ml/min. Mononuclear cells were generally collected at the following flow rates: RBC pump- 9-10 ml/min, plasma pump- 12-14 ml/min, WBC pump- 3.9-4.1 ml/min. Centrifugation speed was 840 rpm. The pump flow rates were adjusted according to dog size. The heavier animals could usually tolerate faster flow rates (30 ml/min). Sodium thiamylal was administered periodically to maintain an anesthetic effect. When approximately 300 ml of leukocyte-rich suspensions were

collected (about 2 hrs), the donor line was removed and the dog's leg was bandaged. The extracorporeal blood was then returned to the dog with approximately 25-40 ml remaining trapped in the separation chamber. Immediately following the leukapheresis procedure 5 ml of protamine sulfate (5 mg/ml, Eli Lilly, Indianapolis, Ind) was administered via the saline drip bag, to neutralize the heparin. An occasional dog (~6%) reacted to the protamine sulfate. Dogs smaller than 9 kg were pre-infused with approximately 250 ml of normal saline to increase the blood volume of the dog and help prevent the donor vein from collapsing.

CFCL concentrates were kept at room temperature and either elutriated within one hour, or directly transfused within two hours of collection, after removal of the erythrocytes.

ERYTHROCYTE REMOVAL FROM CFCL CONCENTRATES

Ficoll-hypaque Sedimentation

Ficoll-hypaque sedimentation was similar to the procedure used by Boyum (1974). The leukapheresis concentrates were diluted 1:1 with Dulbecco's phosphate buffered saline (Gibco Labs., Grand Island, NY) or RPMI Medium 1640 (Gibco Labs, Grand Island, NY). Into 12 50-ml conical tubes were added 24 ml aliquots of diluted concentrate. With a spinal needle, 6 ml of ficoll-hypaque (Ficoll-Paque, Pharmacia Fine Chem., Piscataway, NJ) was carefully placed at the bottom of each tube. Tubes were then centrifuged at 450 x g for 45 minutes. The mononuclear cell layer was removed and the cells were washed by diluting 4:1 with phosphate buffered saline (PBS) or RPMI. Suspensions were then recentrifuged at 200 x g for 20 minutes. Supernatants were removed and the cells were resuspended in 2 gm% albumin in PBS prior to elutriation. Samples were

then taken for CFU-GM, M-CFC, and CFU-F assays, erythrocyte and leukocyte counts, as well as, for differential examination.

Prior to elutriation, human leukocytes were resuspended to a concentration of one million cells/ml in phosphate-buffer saline (PBS) and then passed over a standard Ficoll-Hypaque gradient to obtain mononuclear cell suspensions. Samples were taken for CFU-GM and M-CFC assays, erythrocyte and leukocyte counts, and differential counts.

Dextran Sedimentation

Mononuclear cell loss experienced during ficoll-hypaque sedimentation for removal of erythrocytes was significant. To reduce these losses, dextran sedimentation was subsequently used for CFCL concentrate preparation prior to elutriation on the layer separation chamber. Immediately after leukapheresis, the concentrate was equally distributed into 12 50-ml conical tubes, in volumes of 25 ml each. To each conical tube was then added 2.5 ml of 6 gm% (w/v) dextran T-500 (Pharmacia Fine Chem., Uppsala, Sweden) in normal saline. Suspensions were then mixed thoroughly and erythrocytes were then allowed to sediment at $1 \times g$ for 30 minutes. The top layers which included the leukocytes were collected and combined. The leukocytes were diluted 5-fold with 2 gm% albumin in PBS and centrifuged at $350 \times g$ for 15 minutes on a TJ-6 centrifuge (Beckman Instruments, Palo Alto, CA). Cells were resuspended in 40-100 ml of 2 gm% albumin in PBS prior to elutriation, depending on the size of the elutriation separation chamber used. Samples were taken for CFU-GM, M-CFC, and CFU-F assays, erythrocyte and leukocyte counts, as well as, for differential examinations.

ELUTRIATION MEDIUM

The Ca^{+2} and Mg^{+2} free elutriation medium consisted of 2.0 gm% (w/v) bovine albumin (Fraction V, Sigma Chemical Corp., St. Louis, MO) and 10 mg% EDTA in 0.05 M phosphate buffer plus 0.14 M sodium chloride with a final osmolarity of 325-330 mOsm/kg and a 7.2 pH. The elutriation medium was pressure filtered-sterilized through a 0.20- μm filter assembly (Millipore Corp., Bedford, Mass.) and stored in 1- and 2-liter transfer packs (polyvinyl chloride, PL-146 Travenol plastic, Fenwal Laboratories, Morton Grove, IL).

COUNTERFLOW CENTRIFUGATION ELUTRIATION (CCE-POMEX) PROCEDURE

Smaller 4.3 ml Separation Chamber

The elutriation procedure was similar to the procedure described previously by Contreras, et al. (1980) for the isolation of human lymphocytes and monocytes. Modifications were made in flow rates and in the size of the syringe reservoir. The JE-6 rotor (Beckman Instruments, Palo Alto, CA) speed was maintained at 2020 ± 10 rpm, and the centrifuge temperature was held at 18°C . Approximately 40 ml of a mononuclear leukocyte suspension was entered into the syringe reservoir, consisting of a self-sealing rubber septum with a filtered air vent (Millex-GS, 0.22 μm , Millipore Corp., Bedford, MA) to insure an aseptic environment (Figure 4). Elutriation medium from the 1 or 2 liter transfer pack (MR) was initially directed to the rotor and separation chamber via a rheostat-controlled Sarns cardiovascular pump (Sarns Inc, Ann Arbor, MI) through the mixing chamber pulse suppressor (MCPS) flask. Once the flow rate was established at 8.5 ± 0.3 ml/min for canine studies and 10 ± 0.4 ml/min for human studies, the three-way valve was used to stop the flow from the MR and to initiate

FIGURE 4. Diagram of the aseptic entry of dextran sedimented leukocyte preparations into the JE-6 rotor for the separation of peripheral blood stem cells. The rheostat-controlled Sarns cardiovascular pump provided the fine control of the medium's flow rate essential for the clean separation of peripheral blood committed stem cells from the pluripotent stem cells. MR, medium reservoir; MCPS, mixing chamber pulse suppressor. (Modified from Contreras, et al., J. Cell. Immunol. 54:215, 1980).

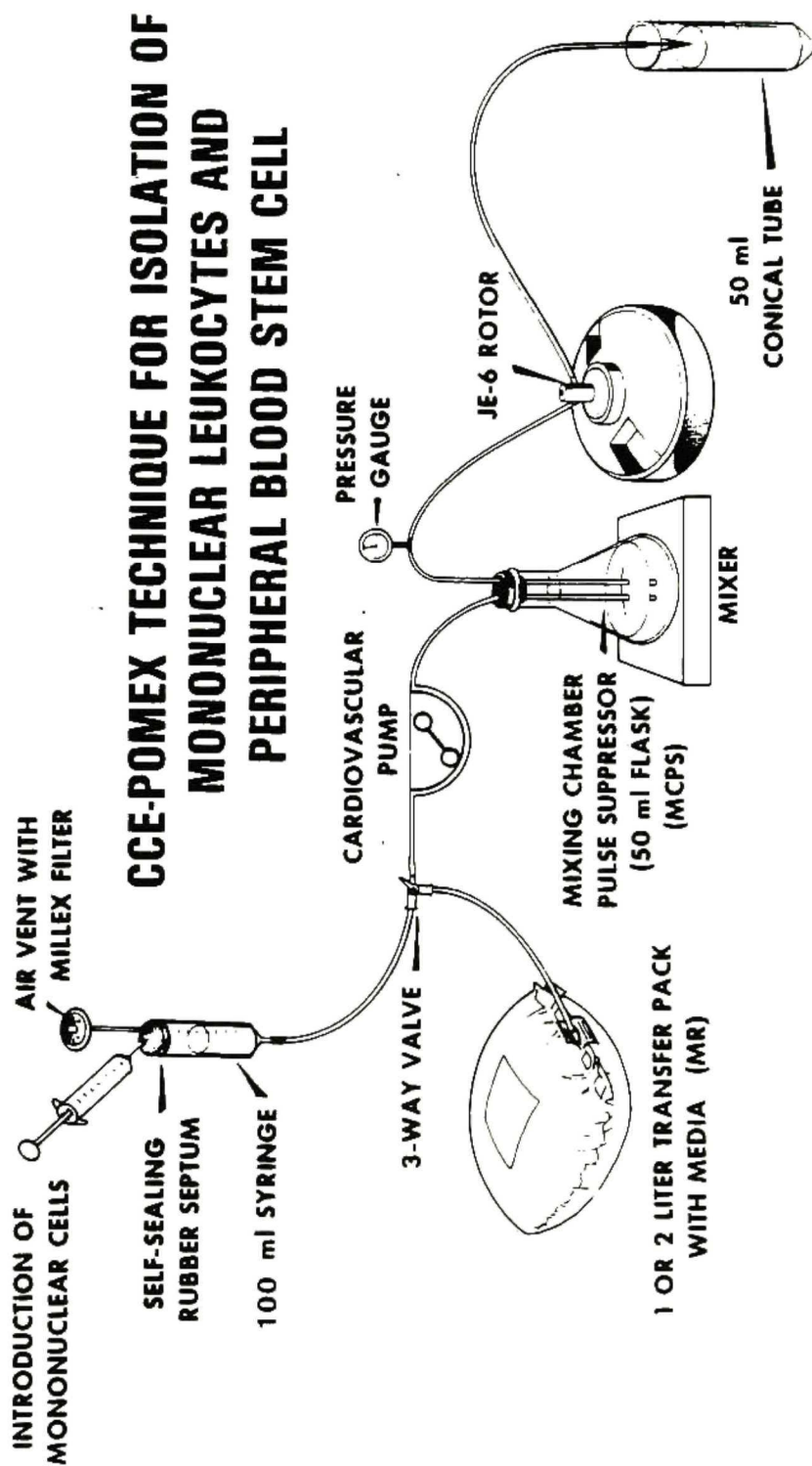


Figure 4

entry of the mononuclear cells. When the cell entry was completed, the three-way valve was again repositioned to resume medium flow from the MR. Four 50-ml aliquots were collected in 50-ml conical tubes (polypropylene, Corning Glass Works, Corning, NY) at the initial 8.5 or 10 ml/min flow rates. The final flow rate of 10.5 ± 0.5 ml/min for canine and 12 ± 0.4 ml/min for humans and the total number of 50-ml fractions collected (10-12 fractions for canine, 9-13 for humans) varied, depending on the elutriation characteristics of each individual mononuclear cell preparation, as well as, the total number of cells entered. To facilitate the graphic representation of these data, all runs were normalized to 12 fractions collected, since 12 fractions was the most frequently occurring number of fractions collected. After the collection of the fourth fraction the flow rate was slowly increased by approximately 0.25 ml/min per additional fraction collected. When an equal number of lymphocytes and monocytes/granulocytes were seen exiting the separation chamber, as determined by the Coulter Channelyzer H4 System (Coulter Electronics, Hialeah, FL) the rotor was stopped but the flow rate was continued. A last 50-ml fraction was collected once the rotor was stopped. This last fraction contained the vast majority of the granulocytes and monocytes present in the initial concentrate. The actual overall elutriation time was 45-60 minutes.

Larger 13.2 ml Separation Chamber

The total number of cells capable of being elutriated on the smaller 4.3 ml separation chamber were insufficient for transplantation studies. Therefore, a larger 13.2 ml separation chamber designed in our laboratory and manufactured at the Armed Forces Radiobiology Research Institute was used. The elutriation procedure, utilizing the larger separation chamber, was primarily the same as for the smaller chamber with the following modifications. Approximately 90-100 ml

of a mononuclear leukocyte suspension was entered in lieu of the 40 ml for the smaller chamber. The initial leukocyte concentrations entered, were about the same for both chambers. The initial flow rate for the larger separation chamber elutriations was 12 ± 0.2 ml/min. Beginning with the fifth 50-ml fraction collected, the flow rate was increased by approximately 0.5 ml/min per additional fraction collected. The final flow rate (16-20 ml/min) and the total number of 50-ml fractions collected (12-20) again depended on the elutriation characteristics of each individual mononuclear cell suspension, and the total number of leukocytes entered into the separation chamber. For convenience of graphic representation all runs were normalized to 14 fractions collected, which was the most frequent number of fraction collected per elutriation. As with the smaller chamber, the rotor was stopped when approximately a 50/50 ratio of lymphocytes to monocytes/granulocytes were exiting the separation chamber. However, two 50-ml fractions were required to remove the remaining leukocytes from the chamber. Again these two last fractions contained the vast majority of the granulocytes and monocytes present in the initial concentrate. The average length of each run on the larger chamber was approximately one hour.

IRRADIATION PROCEDURE

Approximately two hours after leukapheresis, dogs were placed in plexiglas restraint cages while still slightly sedated. Gamma photons from a ^{60}Co radiation source, consisting of four source elements in each cassette, were used for the bilateral exposure. A total dose of 6.0 Gy (600 rad), mid-line tissue dose, was uniformly delivered to the whole body at 0.099 Gy per min (9.9 rad/min). Dose rate measurements were made using an ionization chamber placed along the center-line of a phantom. The free-in-air dose was delivered with a 2-3% precision on all animals. The mid-line tissue dose was determined by the following

equation:

$$\text{Mid-line tissue dose} = [\text{Dose (free-in-air)}][\text{T.A.R.}]$$

The T.A.R. or tissue to air ratio was determined by using a dog phantom simulating the size of dog normally irradiated.

A group of two dogs were exposed to 9.0 Gy in the same manner as described above.

After irradiation exposures, dogs were placed individually in stainless steel cages and either transfused with leukapheresed peripheral blood cells (experimental animals) or with an equal volume of fluid (control animals) within two hours after exposure.

CLONING CULTURE ASSAYS

CFU-GM Procedure

The colony-forming units-granulocyte/macrophage (CFU-GM) derived from the bone marrow and harvested from peripheral blood of dogs, were assayed as previously described by MacVittie and Walker (1980). CFU-GM were assayed using the double-layer agar system with 7% (v/v) plasma from dogs (previously injected with 50 ug endotoxin) as the source of colony stimulating activity (CSA) (MacVittie and Walker, 1978). CMRL 1066 tissue culture media with 10% fetal calf serum, 5% horse serum, supplemented with 30 ug/ml L-asparagine and antibiotics, was made up double-strength for mixing with the agar solutions. Culture medium, in lieu of dog plasma, was used to detect endogenous colony formation that ranged from 0-5 per 2×10^5 nucleated cells cultured. A total of 1×10^6 peripheral blood cells per culture plate were plated in triplicate cultures and

incubated at 37°C in a humidified 5%-CO₂-in-air atmosphere. Colonies of 50 cells or more were counted after 10 days of incubation. Human CFU-GM were assayed using the same procedure as for dog CFU-GM. However, the source of CSA used was medium conditioned by human placenta (HPCM) at 7% (v/v) as described by Burgess, et al. (1977).

M-CFC Procedure

The double-layer agar culture technique, used for the assay of the canine macrophage colony-forming cell (M-CFC) was similar to that described previously by MacVittie and Porvaznik (1978), except that dog serum collected after stimulation with bacterial endotoxin was used as the source of colony-stimulating activity (CSA) (7% v/v) (MacVittie and Walker, 1978). Dog serum was harvested four hours after an intravenous injection (cephalic vein) of 50 ug of endotoxin from Salmonella typhosa (Bacto Lipopolysaccharide W, Difco Laboratories, Detroit, MI) dissolved in pyrogen-free saline.

Peripheral blood cells (PBC) in suspension were plated in triplicate at a concentration of 1×10^6 PBC/dish (35 x 10 mm style, Falcon Labware, Division of Becton-Dickinson and Co., Oxnard, CA) and incubated at 37°C in a humidified, 5% CO₂-in-air atmosphere. Colonies, greater than 50 cells, counted after 27 days were considered to be derived from M-CFC.

Human M-CFC was assayed using the canine assay, with the exception that human placenta conditioned medium (7% v/v) was used as the source of CSA.

CFU-F Procedure

The culture assay used for the quantitation of the colony-forming unit, fibroblast (CFU-F) or as it is also known, the marrow stromal cell (MSC), was similar to the technique used by Wathen et al. (1981). Dog peripheral blood

leukocytes were cultured in 35 mm tissue culture dishes using 2 ml of M-199 containing 3×10^6 nucleated cells per ml. The cultures were incubated at 37°C (5% CO_2 , 95% air humidified atmosphere). On day 3, the medium and suspended cells were aspirated and 2 ml of fresh M-199 was added. On day 14, the cultures were fixed with methanol, stained with Mallory's Azure II Methylene Blue and the adherent colonies of 50 or more cells in each dish were counted.

CFU-M, BFU-E and CFU-E Procedures

Microplasma clot cultures, similar to the procedure of Weinberg et al. (1981), were used to assess megakaryocytic colony-forming unit (CFU-M) activity, as well as, erythroid progenitor cell activity. One-ml cultures consisted of 0.1 ml cell suspensions (100,000 nucleated cells), 0.3 ml fetal bovine serum (heat-inactivated, Rehautin F.S. Reheis Chemical Co., IL., Lot no. T46601 or Flow Laboratories, Inc., Va., Lot no. 29101190), 0.1 ml beef embryo extract (McLeod et al., 1974), 0.1 ml L-asparagine (final concentration 0.02 mg per ml), 0.1 ml 10^{-4} M 2-mercaptoethanol, and 0.1 ml erythropoietin (EPO). Erythropoietin derived from urine of anemic humans served as the source of EPO. For control cultures, supplemented alpha modification of Eagle's Medium (Weinberg et al., 1981) was added instead of EPO. All ingredients were maintained on ice and mixed with 0.1 ml bovine citrated plasma (Gibco, Grand Island, NY) maintained at 37°C , immediately before plating in microtiter wells (sterilized by UV for three hours, Cooke Engineering Co., Va.). For each experimental group, six 0.1-ml microtiter well cultures were established and incubated at 37°C with humidified, 5% CO_2 in air. Three-day cultures were harvested, fixed, stained with benzidine and Geimsa, and examined for aggregates of eight or more benzidine-positive cells, reflected CFU-E (colony-forming unit, erythrocytes). Cultures harvested on day nine with benzidine-stained positive aggregates of more than 100 cells or several clusters of

cells reflected the younger erythroid progenitor cell BFU-E (burst-forming unit, erythrocyte).

Cultures harvested on day seven exhibiting multi-nucleated cell clusters were identified as CFU-M. Pregnant dog uterus conditioned medium was used as the source of thrombopoietin.

EXPERIMENTAL DESIGN FOR CHARACTERIZATION AND AUTOLOGOUS TRANSFUSION OF HEMATOPOIETIC STEM CELLS FROM PERIPHERAL BLOOD

To establish the capability of counterflow centrifugation elutriation to separate hematopoietic stem cells, obtained from peripheral blood, from the immune competent lymphocytes, it was necessary to increase the circulating number of these hematopoietic progenitor cells. A series of animals was given graded doses of dextran sulfate to determine the dosage that would produce the maximum mobilization of stem cells from the bone marrow into the peripheral blood. Mobilization alone was not sufficient to extract the numbers of stem cells required for the characterization of the pluripotent stem cell by autologous transfusions. A continuous flow centrifugation leukapheresis technique, capable of obtaining larger numbers of mononuclear cells from small animals, was established, i.e., CFCL on the IBM 2997 Blood Cell Separator. Bone marrow mobilization, combined with leukapheresis on the IBM 2997, was compared to an existing leukapheresis technique used for larger animals.

Once the maximum peripheral blood stem cell collection from a small dog model had been achieved, CCE fractionation on a 4.3 ml and 13.2 ml separation chamber was maximized. In vitro culture assays of committed stem cells were used to monitor the efficiency of their separation. To establish the characterization of the pluripotent-uncommitted stem cell, the experimental protocol on Figures 5A and 5B was followed. A series of animals was injected

FIGURE 5A. Schematic representation of the experimental protocol for dextran sulfate mobilization, leukapheresis collection, and elimination of red blood cells by dextran sedimentation of peripheral blood stem cells for their characterization and autologous transfusions into lethally irradiated dogs.

Experimental Protocol for Characterization and Autologous Transfusion of Hematopoietic Stem Cells from Peripheral Blood

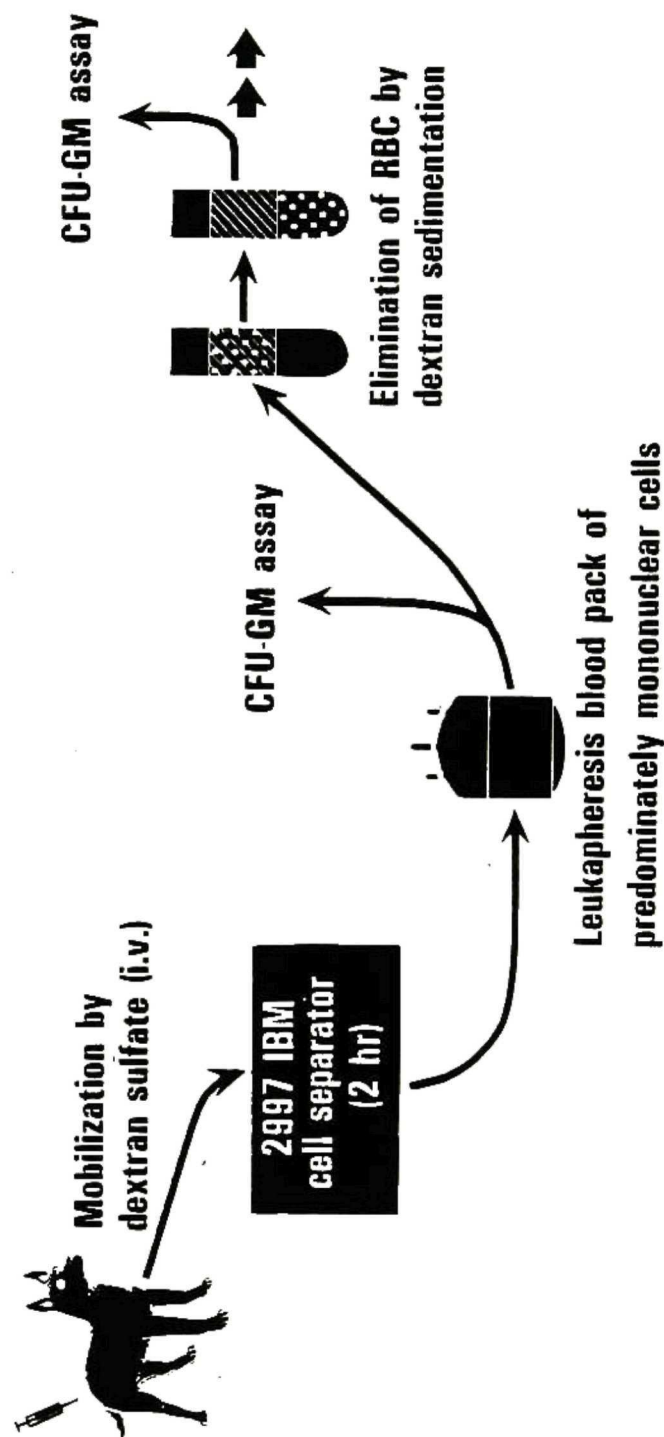
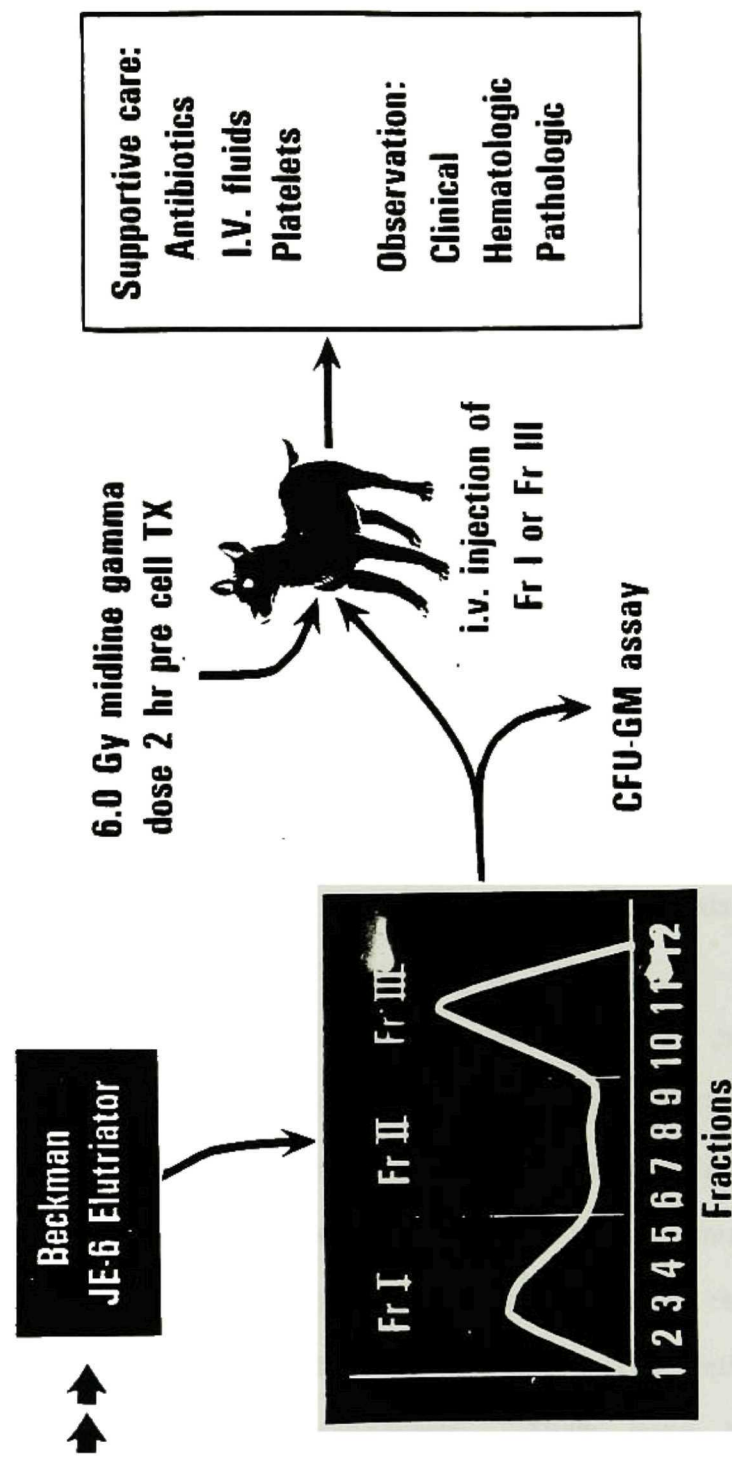


Figure 5A

FIGURE 5B. Schematic representation of the experimental protocol for the counterflow centrifugation elutriation and transfusion of peripheral blood stem cells into lethally irradiated dogs for the characterization of the peripheral blood pluripotent stem cells followed by supportive care and observations.

Experimental Protocol for Characterization and Autologous Transfusion of Hematopoietic Stem Cells from Peripheral Blood



Separation of WBC into subpopulations

Figure 5B

with a double dose of dextran sulfate and then leukapheresed. The leukapheresis concentrate of predominately mononuclear leukocytes, was then assayed for CFU-GM and other committed stem cells, and depleted of erythrocytes. The erythrocyte depleted concentrate was then assayed for CFU-GM, M-CFC, and CFU-F followed by elutriation on the Beckman JE-6 Elutriator. On a series of runs, using both the 4.3 ml and 13.2 ml separation chambers, each 50-ml fraction was assayed for CFU-GM, M-CFC, CFU-M, BFU-E and CFU-E. Total nucleated cells, CFU-GM and M-CFC activities in each fraction were then plotted, with peak values adjusted to 100%. Fractions collected were combined into three major fractions and assayed for CFU-GM and other committed stem cells. Either fraction I or fraction III was then transfused to 6.0 Gy lethally irradiated animals approximately one to two hours after irradiation. The animals were supported with antibiotics, intravenous fluids and platelet transfusion as described later in Materials and Methods section. Animals were also observed clinically, hematologically and pathologically. Necropsies were performed on all decedent dogs to determine the cause of death within 12 hours. All animals were placed in a walk-in 4°C refrigerator within one half hour after death.

A group of three animals was irradiated but not transfused with hematopoietic progenitors cells. These animals were therapeutically supported after irradiation in the identical manner as all other groups of animals.

A series of experiments was performed to establish minimum cellular levels required for the establishment of complete hematopoietic reconstitution of lethally irradiated dogs transfused with peripheral blood stem cells harvested by continuous flow centrifugation leukapheresis. Dogs were transfused with leukapheresis concentrates about one to two hours after irradiation. Transfusion studies of leukapheresis concentrates after erythrocyte removal were also conducted. A set of three dogs received either ficoll-hypaque or dextran

sedimented leukapheresis concentrates, approximately one to two hours after irradiation. Two dogs were transfused with leukapheresis concentrates after being irradiated with 9.0 Gy gamma, to determine the maximum capabilities of these protocols and to deduce additional irradiation damage. Again these dogs were transfused one to two hours after being irradiated.

Fluids

Beginning the day after irradiation all dogs received daily intravenous infusions of 200 ml 50/50 mixture of Ringer's lactate solution and 25% dextrose in 0.45% saline (Abbott, North Chicago, IL), with one ml of multiple vitamin B complex (A.J. Buck, Cockeysville, MD) containing: thiamine-HCl (1 mg), riboflavin (0.05 mg), niacinamide (1 mg), panthenol (0.1 mg), pyridoxine-HCl (0.1 mg), and cyanocobalamide (1 mg). Fluids were given for ten days, longer if clinically warranted.

Food and Water

To reduce gastrointestinal complications all animals were fasted for four days following irradiation and soft food given for an additional four days. Thereafter, animals were fed dry food (Purina Dog Chow, Ralston Purina Co., St. Louis, MO). On occasions certain dogs were also given canned dog food when they were not eating dry food. Tap water was provided ad libitum except immediately after irradiation while the animal was still under the influence of the anesthesia. Water was returned as soon as the animal was over the anesthetic effect.

Antibiotics

Beginning the morning after irradiation, the animals were given 0.75 ml of 40 mg/ml gentamicin sulfate (Garamycin, Schering Corp., Kenilworth, NY)

subcutaneously and 2 ml of 250 mg/ml ampicillin sodium (Polycillin-N, Bristol Labs., Syracuse, NY) intravenously. Antibiotics were given daily until death or until leukocyte counts were greater than 1000 per mm^3 of blood. During the summer months 500 mg of tetracycline HCl (Achromycin, Lederle Lab. Div., Pearl River, NY) were also given daily when rectal temperatures were above 40°C and continued until temperatures returned to normal.

Blood Products

Platelets were obtained from male beagle donors by continuous flow centrifugation plateletpheresis on the IBM 2997 Blood Cell Separator using the dual stage blood separation channel (Figure 2). All dogs were anticoagulated with heparin (0.28 ml/kg) prior to plateletpheresis. Centrifugation speed was 2400 rpm. The RBC pump rate was 14 ml/min, plasma pump rate also 14 ml/min and WBC pump rate was 5-8 ml/min. Anticoagulant pump rate was 4.1 ml/min. Anticoagulant solution used for plateletpheresis was a 50/50 mixture of Anticoagulant Citrated Dextrose Solution-Formula A (ACD-A, Fenwal Labs, Deerfield, IL) and normal saline. Total blood volume processed was approximately 1000 ml. Allogeneic platelet transfusions were given on approximately days 7, 10, 14, and 17 after irradiation to support the animals during the thrombocytopenic episode. All platelets were exposed to 50.0 Gy of gamma photons prior to transfusion to eliminate immunological reactions. Animals dying prior to 14 days were given only two transfusions.

Clinical Observations

Animals were examined every morning for three weeks or until death, to assess their general condition (alertness, dehydration, abscesses, appetite, responsiveness, etc.), administer fluids (10-14 days) or antibiotics, and record deaths. Rectal temperatures were measured every morning.

Laboratory Tests

Complete blood counts and differentials were performed every other day. Platelet counts were done by phase-contrast microscopy. Leukocyte and erythrocyte counts were performed by an electronic particle counter (ZBI, Coulter Electronics, Hialeah, FL). Hematocrits were prepared on the micro-hematocrit centrifuge (Clay Adams, Parsippany, NY). Differential counts were made on air-dried Wrights-Giemsa-stained smears. Necropsy reports on all dogs were prepared by a pathologist of the Department of Veterinarian Medicine.

Statistical Methods

The BMDP biomedical computer program P2V-analysis of variance and covariance including repeated measures was used to compare the influences of various concentrations of dextran sulfate as a mobilization agent on dog peripheral blood CFU-GM, leukocyte, and mononuclear cell concentrations as well as on the differential with repeated measurements over a five hour period. Values of $p < 0.05$ were considered to be statistically significant.

The student's nonpaired t test was used to statistically compare the days of survival between control dogs and those receiving fraction III. The student's nonpaired t test was also used to compare total CFU-GM activity obtained on two leukapheresis instruments and the number of CFU-GM and M-CFC collected from dogs receiving dextran sulfate and those that did not receive dextran sulfate prior to leukapheresis.

Paired t test analyses was used to statistically compare the effect of the administration of 15 mg/kg and 30 mg/kg body weight dextran sulfate on the peripheral blood CFU-GM, leukocyte, and mononuclear cell concentrations before and up to five hours after administration.

RESULTS

I. EVIDENCE FOR THE EXISTENCE OF A BONE MARROW-BLOOD BARRIER IN HUMANS AND CANINES.

Bone Marrow Mobilization Using Dextran Sulfate

Mobilization of bone marrow cells into the peripheral blood was accomplished with the use of the chemical agent dextran sulfate, molecular weight 8,000. Three different concentration studies were conducted to determine the optimal concentration which would result in maximal bone marrow mobilization. The effect of various concentrations per kg body weight, were evaluated. Figure 6A shows that the peripheral blood granulocyte/macrophage committed stem cell, the CFU-GM, increased from a pre-dextran sulfate level of 103 CFU-GM per ml of blood to a high of 1359 CFU-GM per ml of blood three hours after injection of 15 mg per kg of body weight ($p < 0.05$). Mobilization occurred quite rapidly, such that by the end of one hour the CFU-GM concentration had already significantly ($p < 0.025$) increased to 1230 CFU-GM per ml of blood or a 10.2-fold increase (Figure 6B). At five hours after dextran sulfate infusion, the CFU-GM levels were beginning to return toward normal, from a high of 12.0-fold increase at three hours to a 6.1-fold increase. By the end of 24 hours, the peripheral blood CFU-GM levels had returned to normal levels.

Figure 7A presents the influence of 15 mg per kg of body weight dextran sulfate on the white blood cell population. There was an increase from 8.3×10^6 WBC per ml blood to 11.6×10^6 WBC per ml blood at one hour ($p < 0.005$) and 11.9×10^6 WBC per ml blood at two hours after infusion ($p < 0.005$). Again by the fifth hour there was a return toward normal leukocyte blood levels, as seen before in the CFU-GM blood concentrations (Figures 6A and 6B). Dextran sulfate, in this

FIGURE 6.

The influence of 15 mg/kg body weight of dextran sulfate as a mobilization agent on the peripheral blood granulocyte/macrophage progenitor cell (CFU-GM) concentration of dogs. One hour after dextran sulfate administration a significant rise in CFU-GM was observed. By the fifth hour after dextran sulfate administration, a return toward normal levels was observed (A). The increase in CFU-GM at one hour was translated to a 10-fold increase over normal circulating CFU-GM levels (B). Five hours after dextran sulfate administration approximately 6 times the normal CFU-GM circulating baseline levels were still present (*, $p < 0.025$ vs 0 hr; **, $p < 0.05$ vs 0 hr).

**INFLUENCE OF 15 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON DOG PERIPHERAL BLOOD
CFU-GM CONCENTRATIONS**

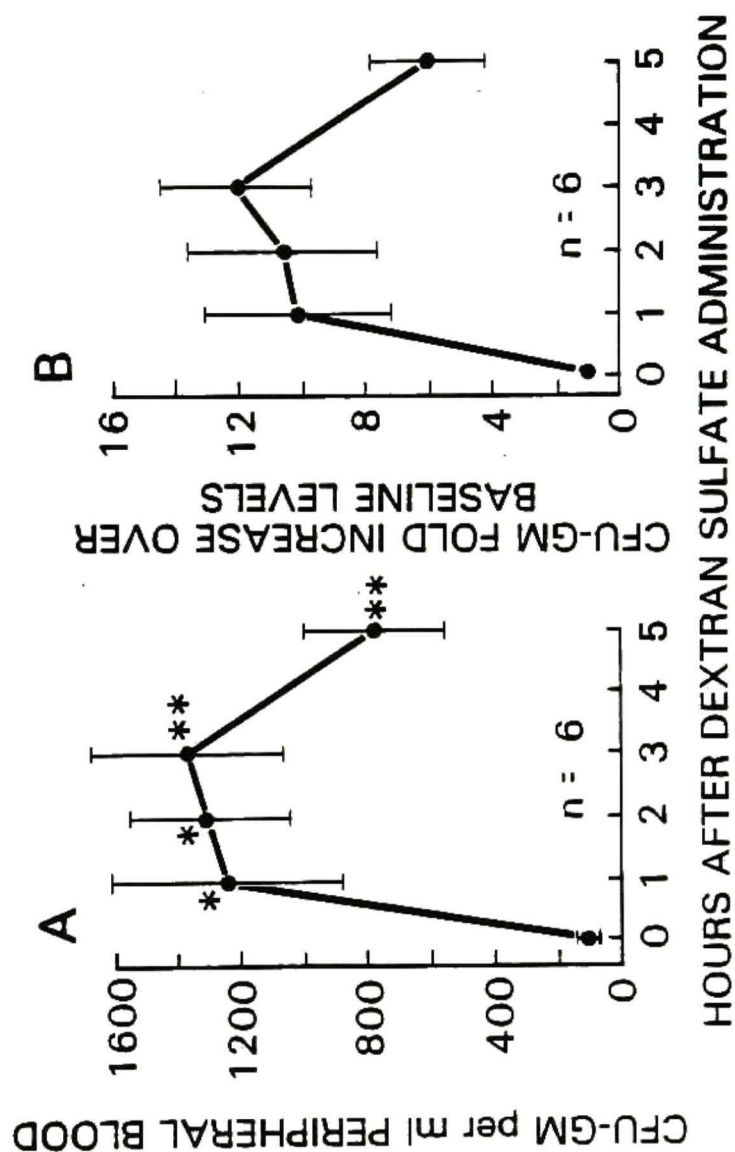


Figure 6

FIGURE 7.

The influence of 15 mg/kg body weight of dextran sulfate as a mobilization agent on the dog peripheral blood leukocyte concentration per ml of blood and factor increase over the baseline levels. A significant rise in circulating white blood cells (WBC) was noted up to 5 hours post dextran sulfate administration. At 2 hours post administration the WBC increase was approximately 4×10^6 per ml (A) or a 1.45 fold increase over normal levels (B). (*, $p < 0.005$ vs 0 hr; **, $p < 0.01$ vs 0 hr).

**INFLUENCE OF 15 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON DOG PERIPHERAL BLOOD
WHITE CELL CONCENTRATIONS**

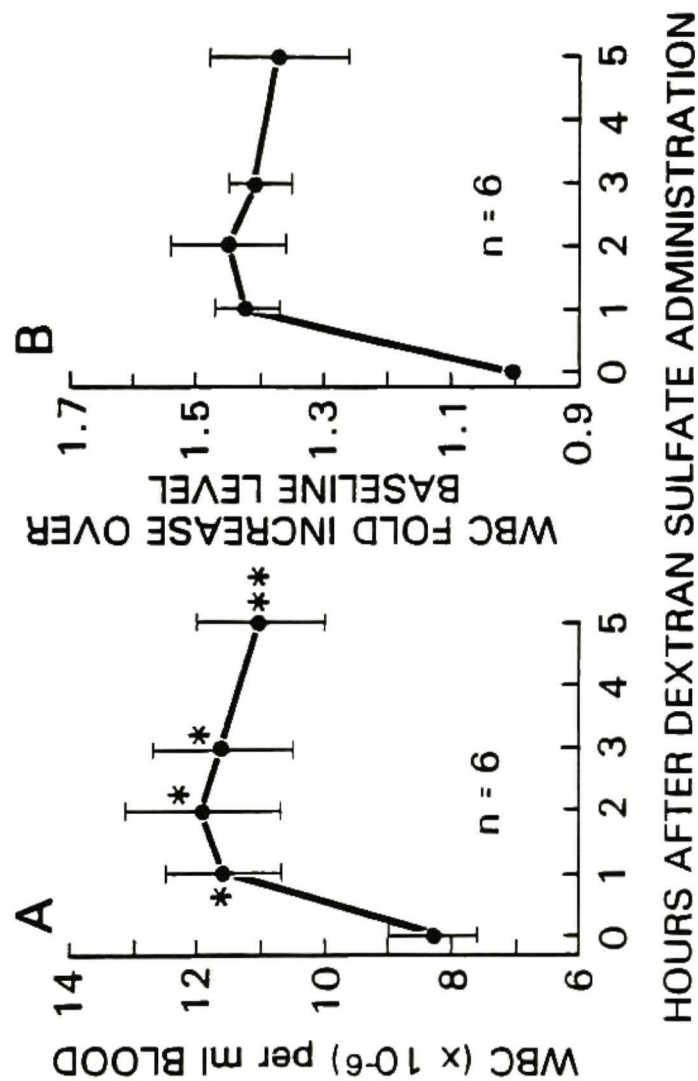


Figure 7

study, caused a 1.40-fold increase in leukocyte concentration for the first three hours (Figure 7B) after injection. This rise in white cell count was predominately due to mobilization of mononuclear cells from extravascular sites (Figures 8A, 8B and 8C). The data in Figure 8A indicates a blood differential shift from 26.8% mononuclear cells (MNC), i.e., lymphocytes and monocytes, at zero hour to 36.2% and 36.8% at one and two hours ($p < 0.005$), respectively, with a gradual return toward normal thereafter. The increase in the percentage of mononuclear cells, along with the increase in total circulating leukocyte count (Figure 7A) resulted in a two-fold enhancement in the number of intravascular mononuclear cells (Figure 8C) from a level of 2.24×10^6 MNC per ml of blood pre-dextran sulfate to 4.21×10^6 and 4.40×10^6 MNC per ml of blood at one and two hours, respectively ($p < 0.005$) (Figure 8B). This surge in peripheral blood mononuclear cells coincides with the rise in the granulocyte-macrophage committed stem cells, implicating the bone marrow as the extravascular source of mononuclear cells.

Concentrations of 7.5 and 30 mg dextran sulfate per kg of body weight, injected into beagles, did not induce the magnitude of CFU-GM mobilization that 15 mg/kg did (Figure 9A and 9B). The results of both the 7.5 and 30 mg/kg dextran sulfate concentration studies were quite similar and to eliminate repetition, only the 30 mg/kg dextran sulfate data is shown. As presented in Figures 9A and 9B respectively, a significant increase from 161 CFU-GM per ml blood at zero hour, to 434 CFU-GM per ml blood at three hours ($p < 0.005$) or only a 3.9-fold increase was evident. Interestingly, however, similar rises in WBC (Figures 10A and 10B) and MNC (Figures 11A, 11B, and 11C) occurred with 30 mg/kg as did with 15 mg/kg dextran sulfate. Concentrations of 7.5 mg/kg dextran sulfate produced less mononuclear cell mobilization than 15 or 30 mg per kg of body weight but the extent CFU-GM mobilization was comparable to that of the 30 mg/kg concentration (data not shown).

FIGURE 8.

The influence of 15 mg/kg body weight of dextran sulfate as a mobilization agent on the dog peripheral blood mononuclear cells in terms of percentage, concentration per ml of blood, and factor increase over baseline levels. The administration of dextran sulfate produced a differential shift in mononuclear cells from a normal circulating level of 27% to as great as 37% at 2 hours (A). This shift in the differential combined with an increase in total circulating WBCs resulted in an increased mononuclear cell number of about 2.2×10^6 per ml of blood (B) or a 2-fold increase above normal levels (C). The increase in circulating mononuclear cell correlated with the increase in circulating CFU-GM levels. (*, $p < 0.025$ vs 0 hr; **, $p < 0.005$ vs 0 hr; ***, $p < 0.05$ vs 0 hr)

**INFLUENCE OF 15 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON PERIPHERAL BLOOD
MONONUCLEAR CONCENTRATIONS**

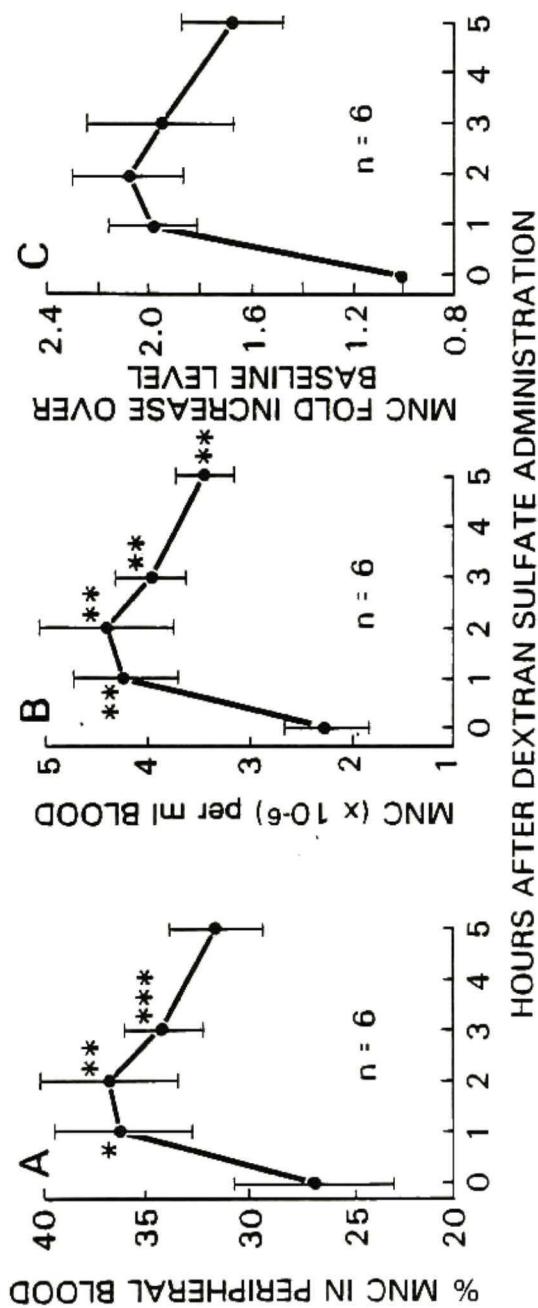


Figure 8

FIGURE 9. The influence of 30 mg/kg body weight of dextran sulfate as a mobilization agent on the peripheral blood granulocyte/macrophage progenitor cell (CFU-GM) concentration of dogs. Administration of 30 mg/kg dextran sulfate resulted in an insignificant increase in CFU-GM at one hour (A) and a maximum 4-fold increase at 3 hours (B). (*, $p < 0.05$ vs 0 hr; **, $p < 0.005$ vs 0 hr).

**INFLUENCE OF 30 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON PERIPHERAL BLOOD
CFU-GM CONCENTRATIONS**

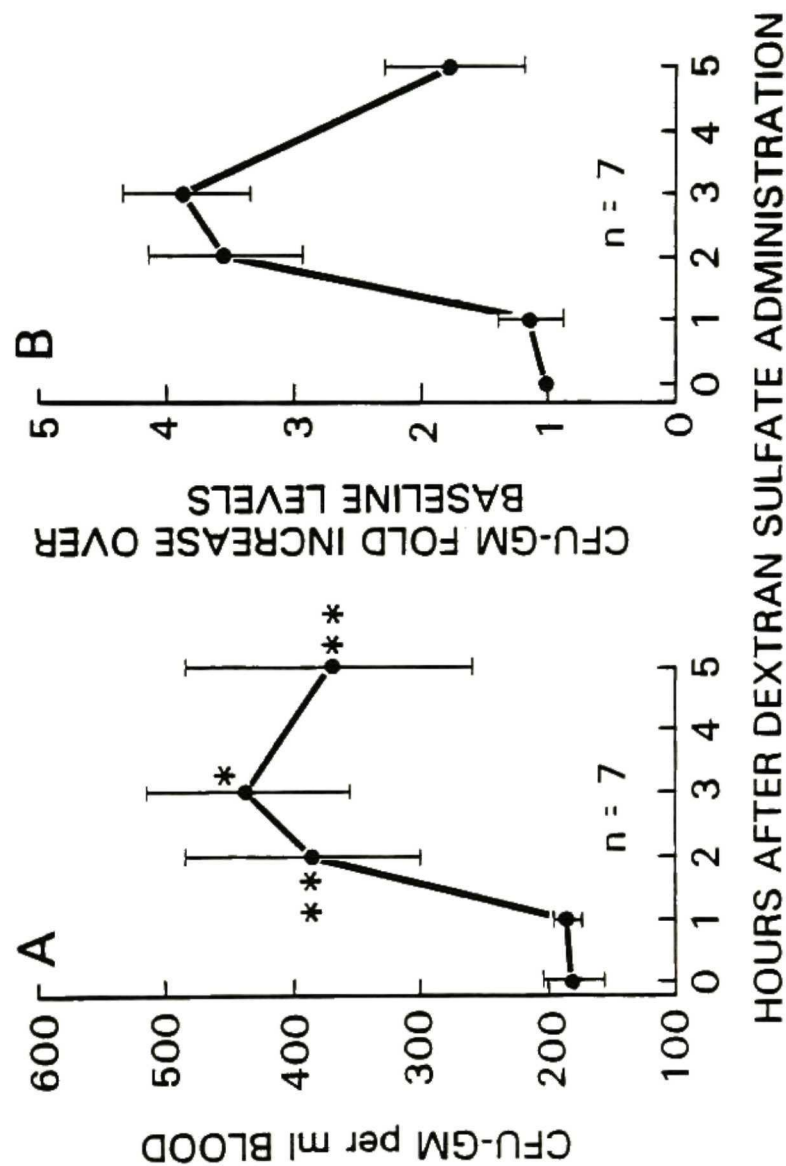


Figure 9

FIGURE 10.

The influence of 30 mg/kg body weight of dextran sulfate as a mobilization agent on the dog peripheral blood leukocyte concentration per ml of blood and factor increase over baseline levels. The mean leukocyte concentration 3 hours after dextran sulfate administration was 12.7×10^6 WBC per ml of blood (A) or a 1.59-fold increase in leukocytes (B). These elevations were greater than those observed with 15 mg/kg dextran sulfate administration. (*, $p < 0.005$ vs 0 hr).

**INFLUENCE OF 30 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON PERIPHERAL BLOOD
WHITE CELL CONCENTRATIONS**

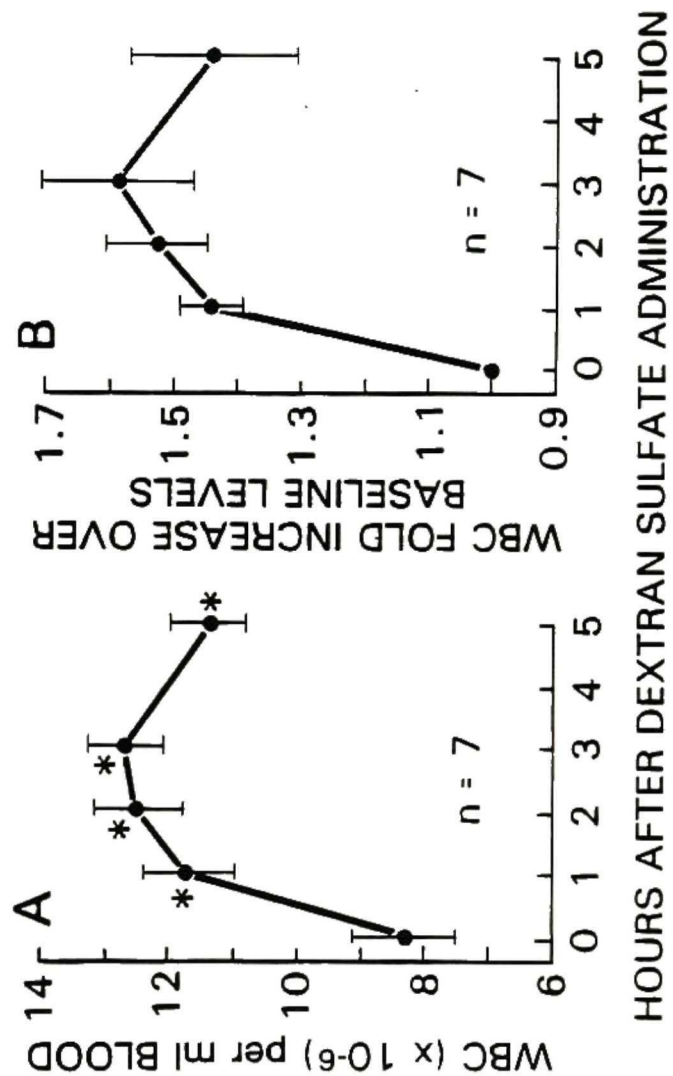


Figure 10

FIGURE 11.

The influence of 30 mg/kg body weight of dextran sulfate as a mobilization agent on the dog peripheral blood mononuclear cells (MNC) in terms of percentage, concentration per ml of blood, and factor increase over baseline levels. A significant differential shift to mononuclear cells was noted in the peripheral blood one hour after dextran sulfate administration, with a rapid shift toward normal within 5 hours (A). This differential shift, in combination with an increase in total circulating leukocytes, resulted in significant increases in circulating mononuclear cells (B). A 2.2-fold increase in circulating MNC was seen at 2 and 3 hours post dextran sulfate administration (C). (*, $p < 0.005$ vs 0 hr; **, $p < 0.01$ vs 0 hr)

**INFLUENCE OF 30 mg/kg DEXTRAN SULFATE AS A
MOBILIZATION AGENT ON PERIPHERAL BLOOD
MONONUCLEAR CELL CONCENTRATIONS**

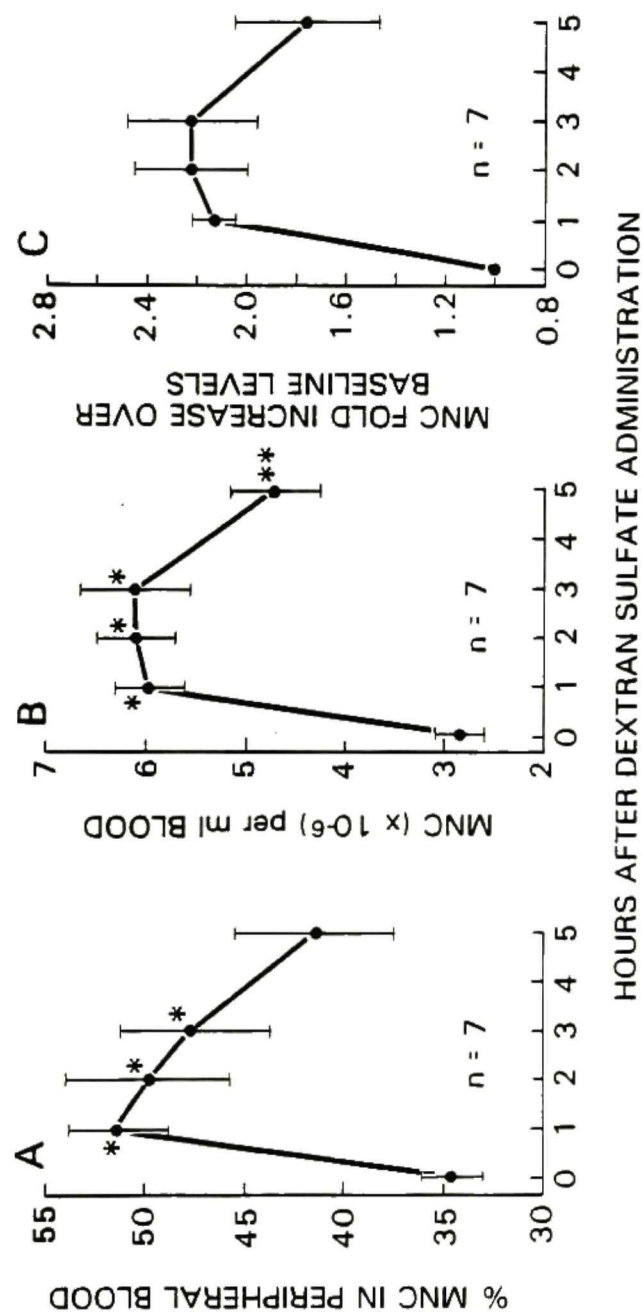


Figure 11

Statistical analysis of variance and covariance including repeated measures revealed a significant effect of dextran sulfate on peripheral blood CFU-GM with respect to various concentrations ($p < 0.006$) and time after administration ($p < 0.001$). The effect of dextran sulfate on leukocyte, mononuclear cell concentrations as well as differentials was significant only with respect to time ($p < 0.001$) and not concentration.

Other evidence of bone marrow cell mobilization was derived from the fact that other committed stem cells, i.e., macrophage precursor cells (M-CFC) and megakaryocyte precursor cells CFU-M, as well as, bone marrow support cells, the stromal cells CFU-F, were present in greater than normal numbers in the CFCL concentrates and CCE fractions.

Peripheral Blood Stem Cell Procurement By Continuous Flow Centrifugation Leukapheresis

Procurement of peripheral blood hematopoietic progenitor cells was accomplished by continuous flow centrifugation leukapheresis (CFCL) (Figure 2). Higher extracorporeal volume requirements on the Aminco Celltrifuge necessitated larger animals (foxhounds) and lower volume collections 266 ± 22 ml ($n=8$) vs 313 ± 13 ml ($n=20$) collected on the IBM 2997 Blood Cell Separator. Although higher leukocyte concentrations were obtained on the Celltrifuge, the mononuclear cell percentage was lower, $60 \pm 9\%$ vs $77 \pm 2\%$ ($\bar{x} \pm S.E.$) for the IBM 2997 Blood Cell Separator. The total number of MNC collected were approximately the same for both instruments. However, the major difference was that the average run-time on the Celltrifuge was approximately four hours (3.84 ± 0.20) while the average run-time on the IBM 2997 Blood Cell Separator was only 2 hours (2.01 ± 0.08). Furthermore, 20 beagles leukapheresed on the IBM 2997 weighed less than half (11.2 ± 0.5 kg) that of eight foxhounds leukapheresed on the

Aminco Celltrifuge (23.7 ± 0.8 Kg). Beagles leukapheresed on the IBM 2997 were given 15 mg dextran sulfate per kg of body weight while foxhounds leukapheresed on the Celltrifuge were not given dextran sulfate. Consequently, there was a 8.8 ± 0.52 -fold increase ($p < 0.005$) in CFU-GM isolation on the IBM 2997. The total CFU-GM activity isolated by CFCL from dextran sulfate stimulated dogs averaged 3.68×10^5 progenitor cells.

Table 1 presents general and technical data obtained from 20 leukapheresis procedures done on the IBM 2997 Blood Cell Separator. The average blood volume processed, 2,430 ml, was approximately three times the dogs' total blood volume.

Erythrocyte Removal From CFCL Concentrates Prior to CCE Fractionation

Prior to CCE fractionation of the peripheral blood stem-cell enriched CFCL concentrates, the red blood cells were removed by either ficoll-hypaque or dextran sedimentation. Red cell removal facilitated the CCE fractionation of stem cells and substantially reduced the length of time of each run. Ficoll-hypaque removed not only erythrocytes but granulocytes as well. Mononuclear cell and CFU-GM losses after ficoll-hypaque sedimentation were 35-55%. The total nucleated cell (TNC) recovery by dextran sedimentation, on the other hand, was 83.5% (Table 2). The CFU-GM recovery was 86.0%. Thus dextran sedimentation for removal of the erythrocytes from CFCL concentrates was far superior to removal by ficoll-hypaque.

Counterflow Centrifugation Elutriation Fractionation of Peripheral Blood Stem Cells Using a 4.3 ml Separation Chamber

Human Studies - Spontaneously Circulating Homogeneous Populations of Committed Stem Cells

TABLE 1.

General and technical data obtained from 20 leukapheresis procedures performed on small beagles on the IBM 2997 Blood Cell Separator. The mean weight of beagles leukapheresed on the IBM 2997 Blood Cell Separator was 11.2 ± 0.5 Kg. The mean weight of eight foxhounds used on the Aminco Celltrifuge was 23.7 ± 0.8 Kg. The average run time on the IBM 2997 was 2.01 ± 0.08 hours compared to 3.84 ± 0.14 hours on the Aminco Celltrifuge.

TABLE 1
GENERAL DATA ON LEUKAPHERESIS PROCEDURE ON THE
IBM 2997 BLOOD CELL SEPARATOR

$n = 20$

	Dog Wt. (kg)	Run Time (hr)	Total Blood Processed (ml)	Time DS Given Prior to CFCL (hr)	Channel Speed (rpm)	Pump Rates (ml/min)		
						RBC	Plasma	WBC
\bar{X}	11.2	2.01	2430	1.09	836	9.3	12.9	4.1
S.E.	0.5	0.08	60	0.10	4	0.7	0.7	0.1

TABLE 2.

Cellular recoveries after dextran sedimentation for removal of erythrocytes from continuous flow centrifugation leukapheresis (CFCL) concentrates prior to counterflow centrifugation elutriation (CCE). These cell recoveries were significantly greater than those obtained by ficoll-hypaque sedimentation ($p < 0.05$). TNC, total nucleated cells; CFU-GM, colony-forming unit, granulocyte/macrophage.

TABLE 2

CELL RECOVERIES POST DEXTRAN SEDIMENTATION

n = 12

	TNC Sedimented ($\times 10^{-9}$)	TNC Recovered ($\times 10^{-9}$)	TNC Recovery (%)	Total CFU-GM Pre Sediment ($\times 10^{-5}$)	Total CFU-GM Post Sediment ($\times 10^{-5}$)	CFU-GM Recovery (%)
\bar{X}	6.97	5.79	83.5	4.20	3.61	86.0
S.E.	0.92	0.78	2.8	0.19	0.42	3.2

Prior studies utilizing the commercially available 4.3 ml counterflow centrifugation elutriation (CCE) separation chamber in the isolation of mononuclear cells (Contreras et al), suggested the possibility of peripheral blood stem cell fractionation. The fractionation was done using the CCE-POMEX (Procedure Of Maximum Extrication) technique (Figure 4). The CCE-POMEX procedure was a modification of our standard CCE procedure used for the purification of large numbers of granulocytes obtained from leukopheresis concentrates (Contreras, et al., 1979). Figure 12 depicts the distribution of nucleated cells, CFU-GM, and M-CFC in each fraction collected. Also indicated is the percent lymphocytes, or morphologically indistinguishable lymphocytic-like cells, present in each fraction. Fraction I was 98-100% lymphocytes with a mean volume of $173 \pm 5 \text{ } \mu\text{m}^3$. Fraction 12 was predominately monocytes ($93.5 \pm 1.3\%$ mean \pm SEM), $3.7 \pm 1.1\%$ lymphocytes and the remaining cells either neutrophils or basophils. Fractions 11 and 12 contained approximately 90% of the CFU-GM activity in all fractions. The average cell volume of fraction III cells was $378 \pm 12 \text{ } \mu\text{m}^3$.

To facilitate the presentation of the data, the results of the 9-13 fractions collected, were combined into three major fractions; I, II, and III (Tables 3 and 4). Normally the data of the first 4 or 5 fractions were combined to form fraction I, data of fractions 6-8 or 5-7 were incorporated into fraction II, depending on the number of fractions collected, and the data of the last three or four fractions were combined into fraction III.

In six experiments, a mean of 2.12×10^9 total nucleated cells were fractionated with a mean recovery of $92.7 \pm 2.8\%$ (mean \pm SEM). Of the total cells entered into the separation chamber and recovered, approximately 54% were isolated in fraction I, 12% in fraction II, and 34% in fraction III (Table 3). About 4.28×10^4 total CFU-GM were present in the concentrates prior to CCE

FIGURE 12. Fractionation by counterflow centrifugation elutriation of human peripheral blood leukocyte, granulocyte/macrophage (CFU-GM) and macrophage (M-CFC) progenitor cells using the 4.3 ml capacity separation chamber and the percentage of lymphocytes in each fraction collected.

HUMAN PERIPHERAL BLOOD STEM CELL FRACTIONATION BY COUNTERFLOW CENTRIFUGATION ELUTRIATION USING THE 4.3 ml SEPARATION CHAMBER

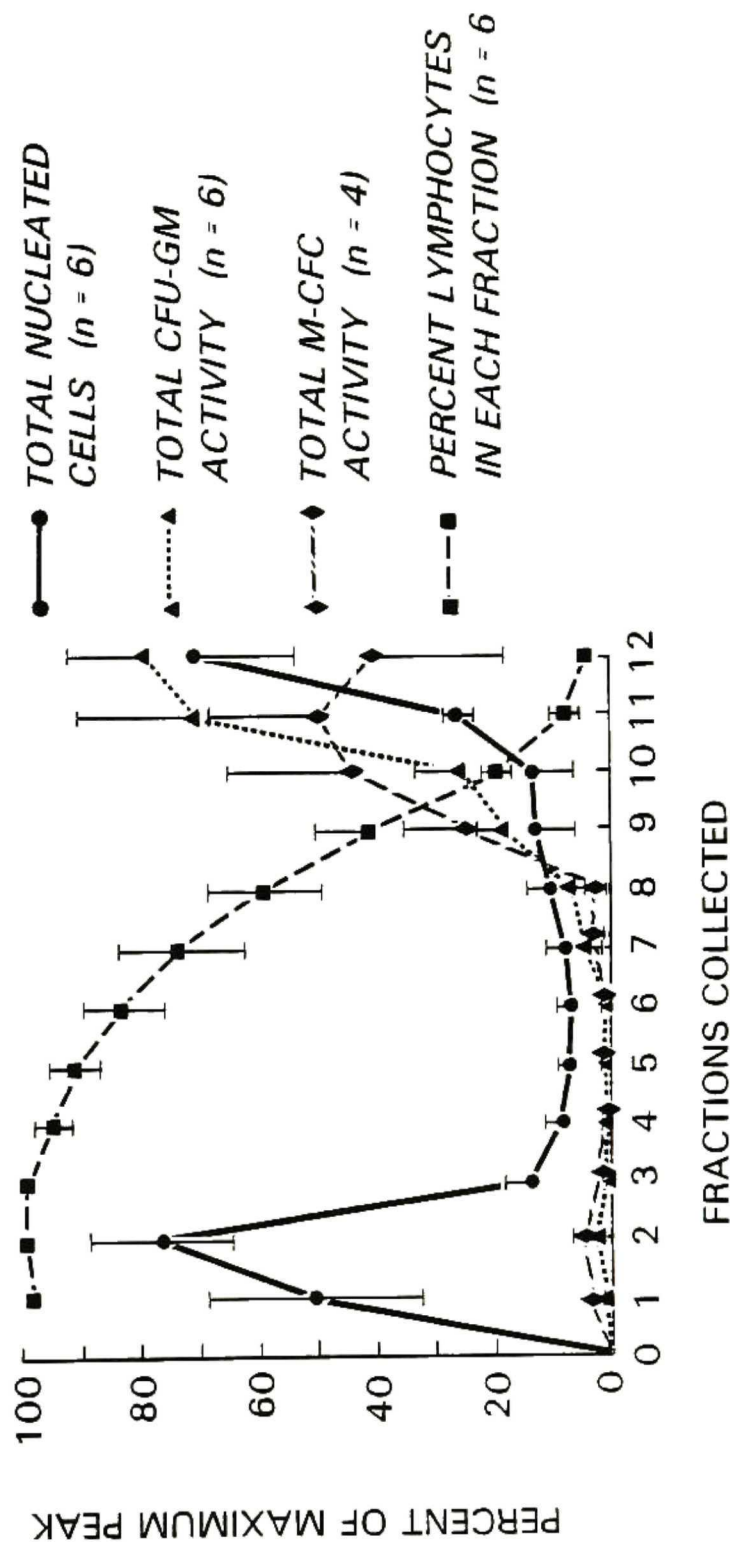


Figure 12

TABLE 3. Fractionation of human peripheral blood nucleated and granulocyte/macrophage progenitor (CFU-GM) cells by counterflow centrifugation elutriation (CCE) on the 4.3 ml capacity separation chamber. The pre fraction was the fraction introduced into the separation chamber for fractionation. (All values are mean \pm S.E.)

TABLE 3

HUMAN PERIPHERAL BLOOD CFU-GM FRACTIONATION USING THE 4.3 ml CCE SEPARATION CHAMBER

n = 6

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	CFU-GM Activity per 10^5 Lymphocytes	Total CFU-GM per Fraction ($\times 10^{-4}$)	CFU-GM per Fraction (%)
Pre	2.12 0.56	100.0 —	2.01 0.71	4.28 1.17	100.0 —
I	1.04 0.21	53.9 12.3	0.08 0.07	0.09 0.11	1.9 2.3
II	0.22 0.05	11.6 2.5	1.90 0.70	0.40 0.21	8.4 4.5
III	0.66 0.20	34.4 10.3	96.70 23.40	4.32 0.84	89.7 6.1

TABLE 4. Fractionation of human peripheral blood nucleated and macrophage progenitor (M-CFC) cells by counterflow centrifugation elutriation (CCE) on the 4.3 ml capacity separation chamber. The pre fraction was the fraction introduced into the separation chamber for fractionation. (All numbers are mean \pm S.E.)

TABLE 4

**HUMAN PERIPHERAL BLOOD M-CFC FRACTIONATION USING
THE 4.3 ml CCE SEPARATION CHAMBER**

n = 4

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	M-CFC Activity per 10^5 Lymphocytes	Total M-CFC per Fraction ($\times 10^{-4}$)	M-CFC per Fraction (%)
Pre	1.86 0.51	100.0 —	2.10 0.70	4.48 1.18	100.0 —
I	0.84 0.13	50.2 13.6	0.26 0.11	0.31 0.15	6.4 3.7
II	0.28 0.06	12.7 1.9	0.82 0.32	0.18 0.11	3.7 1.9
III	0.64 0.14	37.3 7.5	98.00 22.70	4.35 1.01	89.9 5.7

fractionation. Of the CFU-GM activity recovered 2% was localized in fraction I, 8% in fraction II and nearly 90% in fraction III. The initial CFU-GM activity was $2.01 \text{ per } 10^5$ lymphocytes. CFU-GM in fraction III were concentrated approximately 50-fold with respect to CFU-GM activity per 10^5 lymphocytes. The fractionation of the macrophage-colony forming cell, the committed stem cell precursor of the monohistocytic series, the M-CFC, is presented on Table 4. In four experiments, of $90.2 \pm 2.7\%$ (mean \pm SEM). Fraction I contained 50.2% of all the nucleated cells recovered but only 6.4% of all M-CFC recovered. Fraction III, on the other hand, was comprised of 37.3% of the nucleated cells recovered but 89.9% of the total M-CFC recovered. Fraction II contained only 3.7% of the M-CFC activity. Again, there was approximately a 50-fold increase in stem cell activity, i.e., M-CFC activity per 10^5 lymphocytes, in fraction III when compared to the pre-sample (Table 4). The lymphocyte concentration in fraction III of both sets of experiments (CFU-GM and M-CFC) was less than 10% (Figure 12). These data are strongly indicative of homogeneous populations of circulating committed stem cells in humans.

Dog Studies - Spontaneously Circulating and Mobilized Homogeneous Populations of Committed Stem Cells

The above human studies prompted us to investigate stem cell fractionation in a more manageable model, the dog. Leukapheresis concentrates, obtained from dogs that were given 15 mg dextran sulfate per kg of body weight, and those not given dextran sulfate, were ficoll-hypaque sedimented to remove erythrocytes and most granulocytes prior to elutriation on the small 4.3 ml separation chamber. Table 5 shows the stem cell activity in these collections. Nonmobilized-dog concentrates contained 6.2-fold lower CFU-GM activity per 10^5 MNC and 5.5 times fewer total CFU-GM. Mobilized-dog concentrates also

TABLE 5. Peripheral blood- granulocyte/macrophage (CFU-GM) and macrophage (M-CFC) stem cell activities in ficoll-hypaque sedimented leukapheresis concentrates, prior to counterflow centrifugation elutriation (CCE), obtained from dogs not given and those given dextran sulfate to mobilize stem cells from the bone marrow into the peripheral blood. Significantly higher numbers of CFU-GM and M-CFC were collected from dogs receiving dextran sulfate than those that did not ($p < 0.005$).

TABLE 5

**PERIPHERAL BLOOD STEM CELL ACTIVITY IN LEUKAPHERESIS-HYPAGUE
FICOLL CONCENTRATES OBTAINED FROM DEXTRAN SULFATE MOBILIZED AND
NON-MOBILIZED DOGS**

NON-MOBILIZED DOGS

	Total Nucleated Cells ($\times 10^{-9}$)	CFU-GM Activity per 10^5 MNC	Total CFU-GM ($\times 10^{-4}$)	M-CFC Activity per 10^5 MNC	Total M-CFC ($\times 10^{-4}$)
\bar{X}	1.51	0.61	0.96	0.42	0.47
S.E.	0.26	0.14	0.32	0.14	0.15
n	4	4	4	3	3

MOBILIZED DOGS

\bar{X}	1.21	3.76	5.30	2.87	3.04
S.E.	0.27	0.82	1.47	0.66	0.80
n	5	5	5	3	3

contained 6.8-fold greater M-CFC activity per 10^5 MNC and 6.5 times more total M-CFC.

The CCE fractionation of these concentrates yielded combined results similar to those of human fractionation studies (Figure 12). Again, fraction 12 contained very few lymphocytes or morphologically lymphocytic resembling cells ($4.8 \pm 2.0\%$, mean \pm SEM). The predominate cell in fraction 12 was the mature neutrophilic granulocyte and its precursors (60-80%), with a substantial percentage of monocytes (15-35%). Fraction 12 contained approximately 90% of all CFU-GM stem cell activity. As depicted in Figure 13, the macrophage precursor exited the separation chamber slightly ahead of the CFU-GM. On the two occasions that we assayed for the megakaryocytic precursor, the CFU-M, the activity was localized in the last two fractions only. Several experiments were conducted to determine the presence of the burst-forming unit, erythroid (BFU-E) and the colony-forming unit-erythroid CFU-E. Neither progenitor cell was detected in any assay attempted in any elutriated fraction.

As before, to facilitate the presentation of the results, the data of the fractions collected, were combined into three major fractions. These data are presented on Tables 6 and 7. In nine CFU-GM fractionation studies (Table 6) 48% of all nucleated cells recovered were located in fraction I, 16% in fraction II, and 36% in fraction III. However, greater than 91% of the total CFU-GM activity found in all fractions was localized in fraction III, 3.3% in fraction II, and 5.6% in fraction I. The CFU-GM activity was dramatically increased in fraction III to 272 per 10^5 MNC (Table 5). In pre-samples, the vast majority of the MNC were lymphocytes. The mean cell volume of fraction I was $131 \pm 3 \mu\text{m}^3$ and that of fraction III was $255 \pm 8 \mu\text{m}^3$.

Fractionation of M-CFC was even more pronounced (Table 7). Approximately 96% of all M-CFC activity was in fraction III, 1.6% in fraction II

FIGURE 13. Fractionation of canine peripheral blood leukocyte, granulocyte/macrophage (CFU-GM) and macrophage (M-CFC) progenitor cells by counterflow centrifugation elutriation using the 4.3 ml capacity separation chamber and the percentage of lymphocytes in each fraction collected.

CANINE PERIPHERAL BLOOD STEM CELL FRACTIONATION BY COUNTERFLOW CENTRIFUGATION ELUTRIATION ON THE 4.3 ml SEPARATION CHAMBER

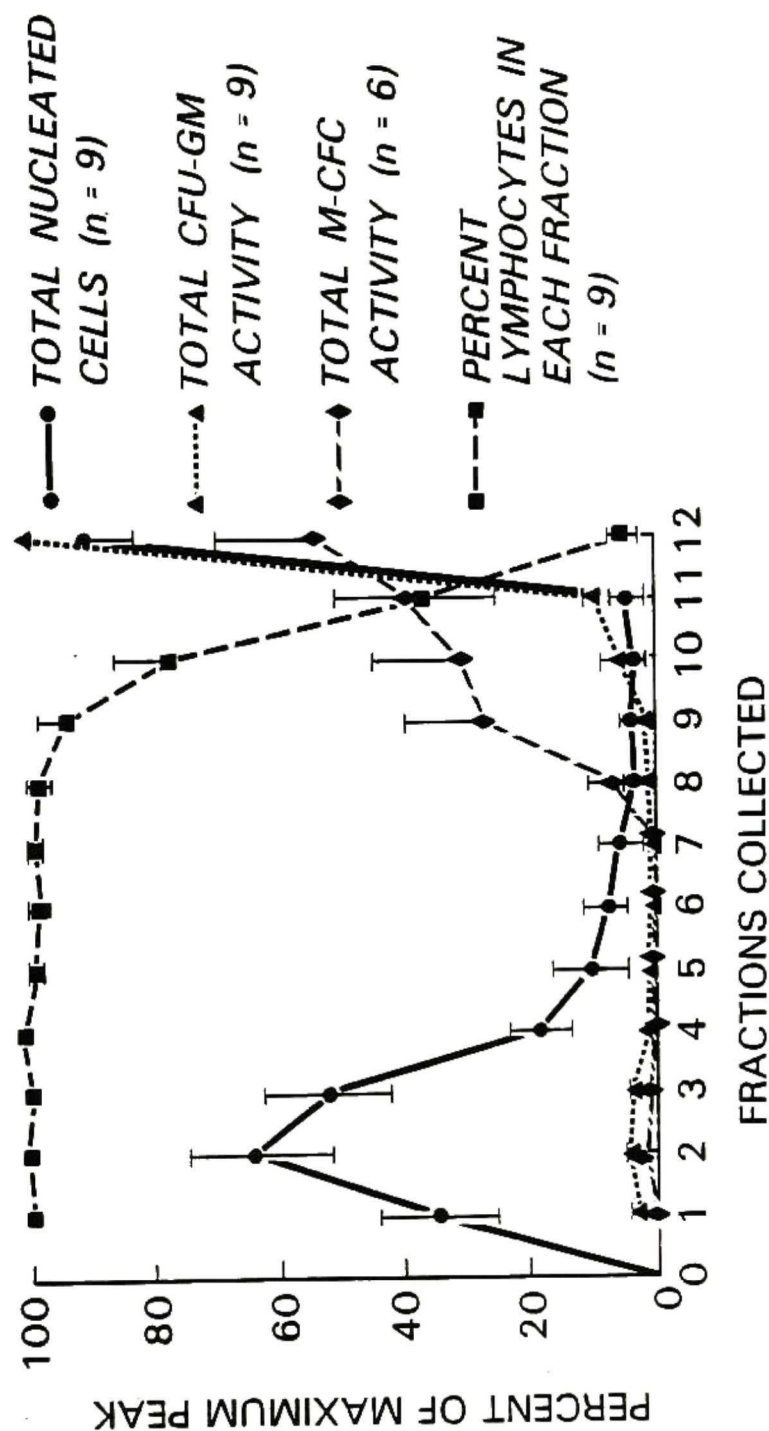


Figure 13

TABLE 6. Canine peripheral blood fractionation of nucleated and granulocyte/macrophage progenitor (CFU-GM) cells by counterflow centrifugation elutriation (CCE) on the 4.3 ml capacity separation chamber. (All numbers are mean \pm S.E.)

TABLE 6

**CANINE PERIPHERAL BLOOD CFU-GM FRACTIONATION USING
THE 4.3 ml CCE SEPARATION CHAMBER**

$n = 9$

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	CFU-GM Activity per 10^5 Lymphocytes	Total CFU-GM per Fraction ($\times 10^{-4}$)	CFU-GM per Fraction (%)
I	0.57	48.1	0.24	0.15	5.6
	0.18	5.8	0.10	0.05	2.0
II	0.18	16.2	0.43	0.08	3.3
	0.03	3.6	0.27	0.05	2.2
III	0.42	36.3	272.10	3.26	91.5
	0.05	3.5	81.20	0.48	3.5

TABLE 7. Canine peripheral blood fractionation of nucleated and macrophage progenitor (M-CFC) cells by counterflow centrifugation elutriation (CCE) on the 4.3 ml capacity separation chamber. (All numbers are mean \pm S.E.)

TABLE 7

**CANINE PERIPHERAL BLOOD M-CFC FRACTIONATION USING
THE 4.3 ml CCE SEPARATION CHAMBER**

$n = 6$

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	M-CFC Activity per 10^5 Lymphocytes	Total M-CFC per Fraction ($\times 10^{-4}$)	M-CFC per Fraction (%)
I	0.45 0.07	40.7 2.2	0.17 0.13	0.08 0.05	2.7 2.1
II	0.21 0.05	20.2 2.9	0.23 0.14	0.04 0.04	1.6 1.5
III	0.44 0.09	39.2 4.1	120.70 22.40	2.29 0.64	95.8 3.5

and 2.7% in fraction I. This difference is due to the smaller amount of M-CFC spillage into the earlier fractions (Figure 13). These spillages occurred in those runs where the capacity of the separation chamber was taxed and/or the initial flow rates or cellular concentrations were too high. Again, as previously seen with the CFU-GM, the M-CFC activity was greatly concentrated in fraction III. The M-CFC activity was 121 M-CFC per 10^5 lymphocytes. The original concentration was 0.42 - 2.87 M-CFC per 10^5 mononuclear cells (Table 5). The pre-sample's mononuclear cell population was predominately lymphocytes.

These data, as those of the human studies, show the presence of homogeneous populations with respect to density, size, volume, of CFU-GM, M-CFC, plus CFU-M circulating in dog peripheral blood. Data obtained from peripheral blood of dogs not given and those dogs given dextran sulfate, were combined without significant change in the results. These results indicate that the circulating committed stem cell populations were identical in both stimulated and unstimulated dogs.

Counterflow Centrifugation Elutriation of Peripheral Blood Stem Cells Employing a Larger 13.2 ml Separation Chamber

Dog Studies - Mobilized Homogeneous Populations of Circulating Committed Stem Cells

The total number of cells capable of being fractionated with the smaller separation chamber was not sufficient for bone marrow engraftment studies due to its limited capacity. Thus a larger 13.2 ml chamber was examined for its potentiality in separating peripheral blood hematopoietic progenitor cells. Results using the larger separation chamber are shown on Figure 14 and Tables 8-10.

The fractional distribution of nucleated cells on the 13.2 ml separation

FIGURE 14. Fractionation of canine peripheral blood leukocyte and granulocyte-macrophage (CFU-GM) committed stem cells by counterflow centrifugation elutriation using the 13.2 ml capacity separation chamber and the percentage of mononuclear cells in each fraction collected.

**CANINE GRANULOCYTE-MACROPHAGE COMMITTED STEM CELL
FRACTIONATION BY COUNTERFLOW CENTRIFUGATION ELUTRIATION
USING THE 13.2 ml SEPARATION CHAMBER**

n = 10

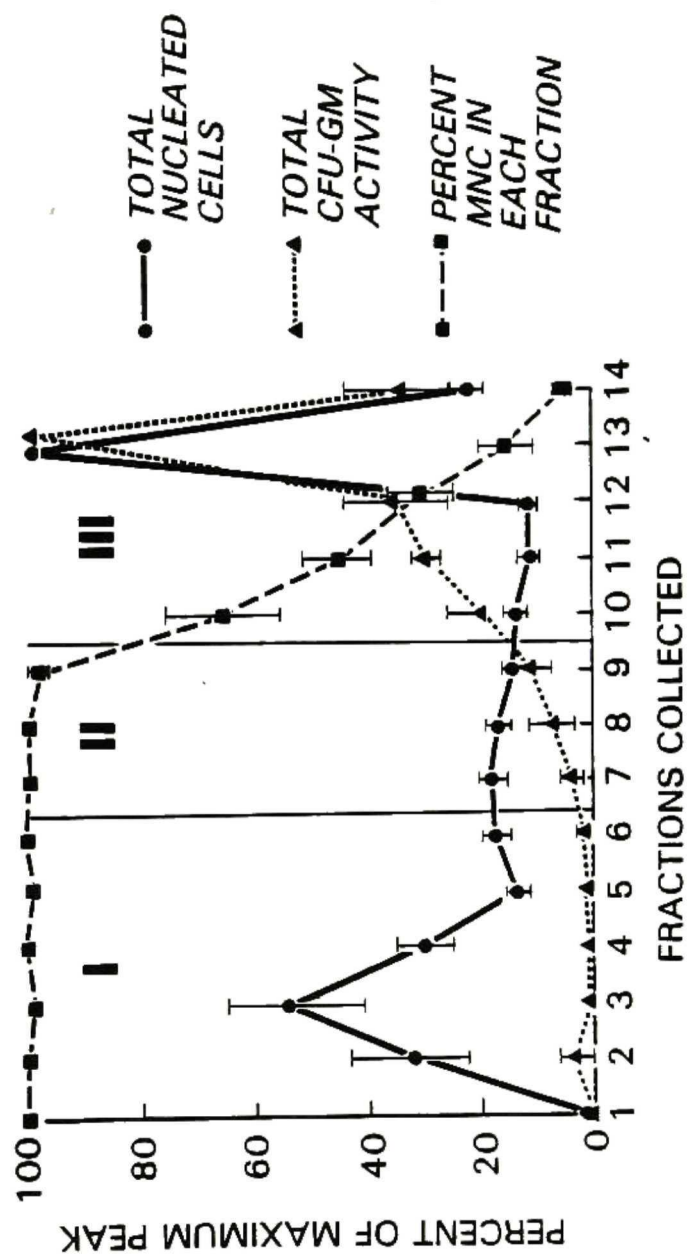


Figure 14

chamber, was basically a bimodal curve (Figure 14). Its configuration resembled that of the nucleated cell distribution of the smaller chamber (Figure 13), with the exception that the last fraction was not, in this case, the maximum peak. To insure that all the cells exited the larger chamber, once the rotor was stopped, one more fraction was collected beyond that required on the smaller chamber. Due to the greater capacity of the chamber and to lower effective entry cellular concentration, less CFU-GM spillage was noted in the earlier fractions as compared to the smaller chamber. As illustrated in Figure 14, the vast majority of the CFU-GM activity was again located in the last fractions.

Table 8 presents general data obtained from 15 dog-mononuclear cell fractionations on the enlarged chamber. Of most significance, was the excellent recovery of approximately 90% of all cell entered.

In previous studies, using the smaller chamber, fractional data was combined to facilitate data presentation. In these studies, however, fractions were physically combined and assayed. Figure 14 shows the approximate demarcations of the fractional pooling. Table 9 summarizes the fractionation of the CFU-GM committed stem cell on the larger 13.2 ml separation chamber. The distribution of total nucleated cells was similar to the cellular distribution of the smaller chamber. Fraction I contained only 2.5% of all CFU-GM activity, fraction II contained 6.7% and fraction III had a mean of 90.8% of the CFU-GM activity present in all three fractions. In terms of CFU-GM activity per 10^5 mononuclear cells, fraction III contained a 279-fold greater concentration of the granulocyte-macrophage precursor cell than did fraction I and a 58-fold greater total CFU-GM. The CFU-GM activity in fraction III was 189 ± 40 CFU-GM per 10^5 lymphocytes. Fraction I consisted of 98-100% mononuclear cells of which all were lymphocytes, or morphologically lymphocytic resembling cell types, while fraction III consisted of 4-20% mononuclear cells of which the vast majority were

TABLE 8.

Canine nucleated cell recovery after counterflow centrifugation elutriation (CCE) using an enlarged 13.2 ml capacity separation chamber. Of special note was the excellent recovery of 90.2% of all nucleated cells introduced into the separation chamber.

TABLE 8
CELL RECOVERY OF ENLARGED 13.2 ml CCE SEPARATION CHAMBER

$n = 15$

	Volume Entered (ml)	Concentration ($\times 10^{-6}/\text{ml}$)	Total Nucleated Cell Entered ($\times 10^{-9}$)	Total Nucleated Cell Recovered ($\times 10^{-9}$)	Recovery (%)
\bar{x}	91.2	54.6	4.90	4.49	90.2
S.E.	5.4	6.3	0.70	0.68	0.2

TABLE 9. Fractionation of canine peripheral blood granulocyte/macrophage progenitor (CFU-GM) and nucleated cells by counterflow centrifugation elutriation (CCE) using the enlarged 13.2 ml capacity separation chamber and the mononuclear cell content of each fraction. (All numbers are mean \pm S.E.)

TABLE 9

**DOG PERIPHERAL BLOOD CFU-GM FRACTIONATION USING A
13.2 ml CCE SEPARATION CHAMBER**

n = 15

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	CFU-GM Activity per 10^5 MNC	Total CFU-GM ($\times 10^{-5}$)	CFU-GM per Fraction (%)	Mononuclear Cells per Fraction (%)
Pre	4.89 0.70	100.0 —	8.5 1.6	3.22 0.61	100.0 —	70 - 84
I	2.19 0.33	49.9 4.4	0.3 0.1	0.06 0.04	2.5 1.9	98 - 100
II	0.48 0.18	10.4 2.2	5.7 3.2	0.19 0.04	6.7 1.8	80 - 95
III	1.77 0.33	39.7 3.0	83.7 16.7	3.46 0.48	90.8 3.0	4 - 20

granulocytic precursor cells, i.e., promyelocytes, myelocytes, and metamyelocytes. The mean cell volume of fraction I was $135 \pm 4 \text{ } \mu\text{m}^3$ and that of fraction III was $260 \pm 7 \text{ } \mu\text{m}^3$.

Dog peripheral blood M-CFC stem cell fractionation, using the 13.2 ml CCE separation chamber, is presented on Table 10. As previously seen in the fractionation of M-CFC on the smaller chamber (Table 7), the fractionation on the larger chamber was just as impressive. Although, 46.7% of the recovered nucleated cells were present in fraction I, only 1.2% of the recovered M-CFC were localized in fraction I. Fraction III, on the other hand, consisted of 41.9% of the recovered nucleated cells and 91.3% of all M-CFC recovered from all three fractions. M-CFC activity per 10^5 MNC in fraction III was concentrated 15-fold over the pre-elutriation value and was over 500-fold more concentrated in M-CFC per 10^5 MNC than fraction I. Fraction III also had a 82-fold greater content of total M-CFC than fraction I.

A double dextran sulfate regimen that increased bone marrow cell mobilization (see Materials and Methods) and a larger number of cells collected during CFCL, accounted for the order of magnitude greater number of CFU-GM and M-CFC.

Fractionation of bone marrow "permissive hematopoietic microenvironmental" cells - the stromal cells is presented on Table 11. The assayed stromal cells more recently termed the bone marrow fibroblast colony-forming cells (CFU-F) were present in CFCL concentrates in two orders of magnitude lower numbers than CFU-GM and M-CFC (Tables 9 and 10). CFU-F fractionation was quite similar to that of CFU-GM and M-CFC. Fraction I demonstrated only 1.9% of the CFU-F recovered, fraction II contained 5.2%, and fraction III consisted of 92.9% of all the CFU-F recovered. The CFU-F activity per 10^6 MNC, in fraction III, was 18.7 times greater than the pre-elutriation

TABLE 10. Fractionation of canine peripheral blood macrophage progenitor (M-CFC) and nucleated cells by counterflow centrifugation elutriation (CCE) using the enlarged 13.2 ml capacity separation chamber and mononuclear cell content of each fraction. (All numbers are mean \pm S.D.)

TABLE 10

**DOG PERIPHERAL BLOOD M-CFC FRACTIONATION USING
THE 13.2 ml CCE SEPARATION CHAMBER**

$n = 2$

Fraction	Total Nucleated Cells ($\times 10^{-9}$)	Nucleated Cells per Fraction (%)	M-CFC Activity per 10^5 MNC	Total M-CFC per Fraction ($\times 10^{-5}$)	M-CFC per Fraction (%)
Pre	4.41 0.53	100.0 —	6.9 1.4	2.34 0.55	100.0 —
I	1.85 0.27	46.7 3.9	0.2 0.2	0.03 0.03	1.2 0.4
II	0.42 0.15	11.1 1.2	6.1 3.7	0.21 0.06	7.6 2.9
III	1.65 0.30	41.9 3.4	106.0 26.0	2.47 0.51	91.3 2.9

TABLE 11. Counterflow centrifugation elutriation (CCE) of the canine bone marrow stromal precursor cell or colony forming-unit, fibroblast (CFU-F) on the enlarged separation chamber. (All numbers are mean \pm S.E.)

TABLE 11

BONE MARROW STROMAL CELL FRACTIONATION BY CCE

n = 8

Fraction	CFU-F Activity per 10^6 MNC	Total CFU-F ($\times 10^{-3}$)	% of Total in Each Fraction
Pre	1.27	5.40	100.0
	0.25	1.44	—
I	0.04	0.10	1.9
	0.04	0.11	0.8
II	1.15	0.25	5.2
	1.06	0.18	1.4
III	23.70	6.24	92.9
	5.40	1.67	4.0

sample and 95 times greater than fraction I. The total number of CFU-F in fraction III was 62 times greater than in fraction I.

The above results indicate the presence of homogeneous circulating populations of CFU-GM and M-CFC as well as CFU-F in dogs, as demonstrated by use of the larger elutriation separation chamber. These data, obtained from dogs receiving dextran sulfate, were very similar to those of dogs who did not receive dextran sulfate. Again, indicating that the homogeneous populations obtained from both groups are identical.

II. PERIPHERAL BLOOD TRANSFUSIONS OF LETHALLY IRRADIATED DOGS TO ASCERTAIN THE PHYSICAL SEPARATION OF PLURIPOTENT AND COMMITTED STEM CELLS

CCE fractionation of the committed hematopoietic stem cells, CFU-GM, M-CFC, CFU-M, plus the stromal fibroblast cell, CFU-F, had been successful in the separation of these progenitor cells from the vast majority of the immune competent lymphocytes. Evidence of the fractionation characteristics of the dog pluripotent stem cell had not been obtained from these studies. The direct assay for the dog pluripotent stem cell has been very difficult. To date no assay technique has been successful in the direct quantification of the dog pluripotent stem cell. However, the presence of the pluripotent stem cell is essential for the complete hematopoietic recovery of lethally irradiated bone marrow. Thus an in vivo approach to the determination of the presence of the pluripotent stem cell was undertaken. This was accomplished by the autologous transfusion of CCE fractionated peripheral blood cells into lethally irradiated dogs.

Negative Control - Non Transfused Dogs

A group of three dogs were lethally irradiated with 6.0 Gy (midline tissue dose), but not transfused. Death ensued 11 to 13 days after irradiation of classical hematopoietic death syndrome, with no pathological evidence of gastrointestinal syndrome involvement. Figure 15 shows the erythrocyte counts of these control dogs. Dogs No. 2 and 3 showed a sudden increase in erythrocyte count and an elevated hematocrit just prior to death, indicative of marked dehydration. Figure 16 depicts the white blood cell (WBC) count of the negative control dogs. Neutropenia, i.e., granulocyte counts of less than 500 per mm^3 (5×10^5 per ml) of blood, occurred 5 to 6 days following irradiation and persisted till death. One day

FIGURE 15. Peripheral blood erythrocyte counts of control beagles exposed to 6.0 Gy mid-line tissue dose of gamma photons and not transfused with peripheral blood cells. A volume of a 50/50 mixture of Ringers lactate and 25% dextrose in 0.45% saline was given equal to the volumes of peripheral blood concentrates given to the experimental animals.

ERYTHROCYTE COUNTS OF LETHALLY IRRADIATED DOGS NOT TRANSFUSED [NEGATIVE CONTROLS]

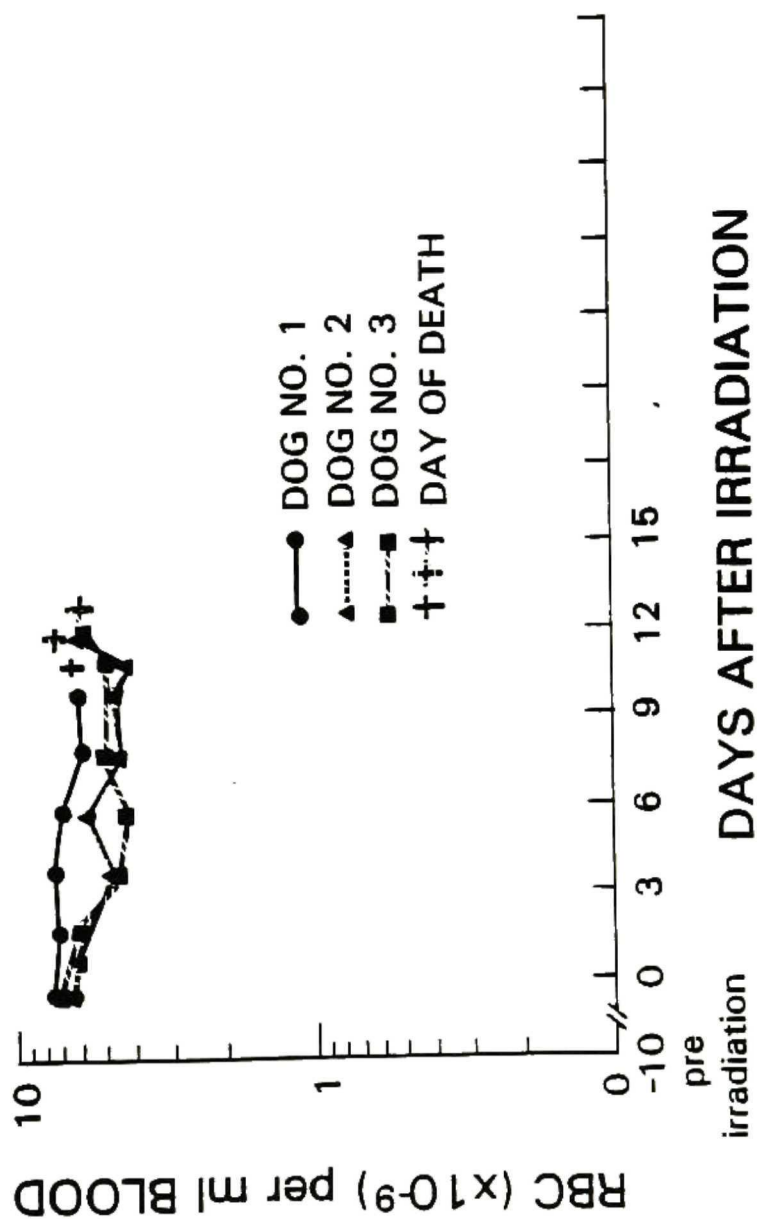


Figure 15

FIGURE 16. Peripheral blood leukocyte counts of control beagles exposed to 6.0 Gy midline tissue dose of gamma photons and not transfused with peripheral blood concentrates.

WBC COUNTS OF LETHALLY IRRADIATED DOGS NOT TRANSFUSED [NEGATIVE CONTROLS]

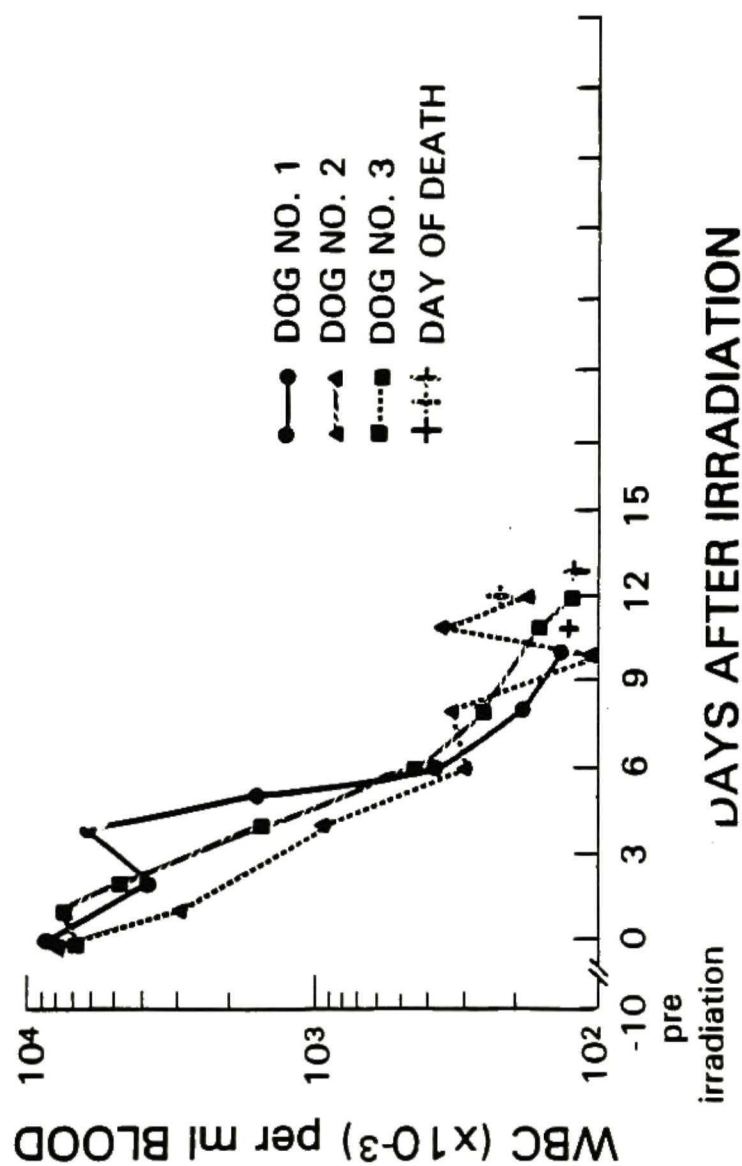


Figure 16

after irradiation, a sharp drop in the radiosensitive lymphocytes was noted in the peripheral blood. Severe thrombocytopenia, platelet counts of less than ten thousand per mm^3 of blood, was noted 10 to 12 days after irradiation in dogs No. 2 and 3, despite platelet transfusions on two separate occasions (Figure 17). Dog No. 1 died prior to becoming severely thrombocytopenic. The therapeutic support of these dogs was the same as in all transfused groups.

CFCL Concentrate Transfused Dogs

Knowing the limitations of peripheral blood stem cell procurement, transfusions were performed to determine the feasibility of bone marrow engraftment of lethally irradiated dogs with the limited number of pluripotent stem cells obtained on the IBM 2997 Blood Cell Separator. Table 12 presents the data obtained from eight CFCL concentrate transfusions. Dogs No. 4 and 5, irradiated with 6.0 Gy, received a mean of 5.05×10^8 MNC/kg containing 1.82×10^4 CFU-GM/kg of body weight (Table 13). Both dogs achieved complete leukocyte recovery, with normal differentials, within 40 days following irradiation and transfusion (Figure 18). Red cell counts were back to normal within 60 days. The abrupt drop in RBC count 24 hours after irradiation and transfusion (Figure 19), was due to bleeding from the intravenous sites, following leukapheresis. Despite this blood loss, erythrocyte recovery was complete. A severe thrombocytopenic episode occurred 10-14 days following irradiation, despite four platelet transfusions (Figure 20). Blood platelet levels were back to within normal levels approximately 55 days after irradiation.

Dog No. 14 was the recipient of a CFCL concentrate of low MNC recovery and poor CFU-GM activity (Table 12). These cellular levels were insufficient for a successful bone marrow engraftment. Subsequently, the dog died on day 14 following irradiation of symptoms consistent with hematopoietic

FIGURE 17. Peripheral blood platelet counts of control beagles exposed to 6.0 Gy mid-line tissue dose of gamma photons and not transfused with peripheral blood concentrates.

PLATELET COUNTS OF LETHALLY IRRADIATED DOGS NOT TRANSFUSED [NEGATIVE CONTROLS]

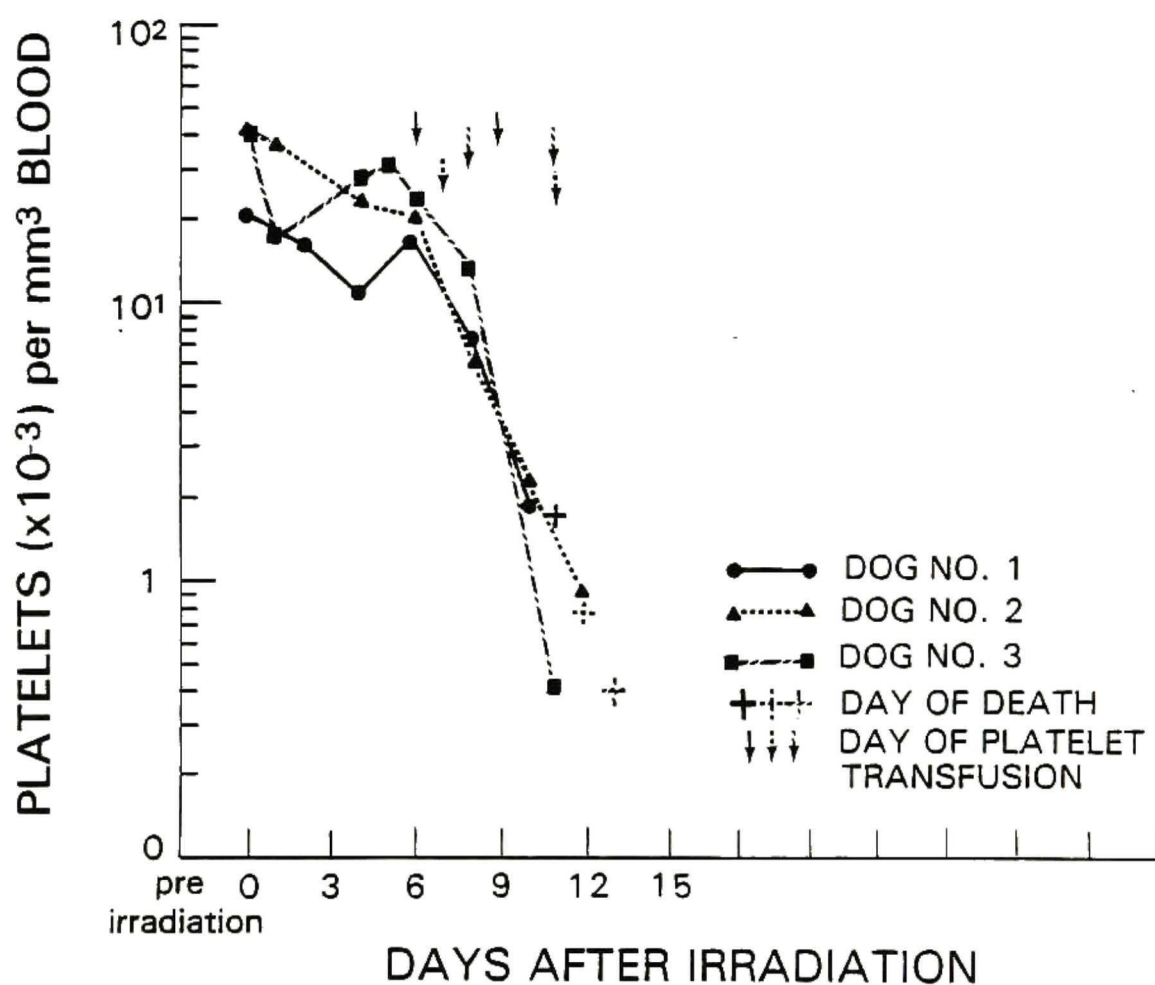


Figure 17

TABLE 12.

Transfusions of continuous flow centrifugation leukapheresis (CFCL) concentrates to determine the minimum cellular requirements for the survival of dogs exposed to 6.0 Gy and 9.0 Gy mid-line tissue dose of gamma photons.

TABLE 12

**TRANSFUSION OF CFCL CONCENTRATES TO
DETERMINE MINIMUM CELLULAR REQUIREMENTS
FOR SURVIVAL OF LETHALLY IRRADIATED DOGS**

Dog No.	Transfused Cells per kg Body Wt.		Survival (days)	Gamma Dose (Gy)
	MNC ($\times 10^{-8}$)	CFU-GM ($\times 10^{-4}$)		
4	4.20	2.10	>294 Alive	6
5	5.90	1.53	>280 Alive	6
7	5.00	0.84	>174 Alive	6
8	1.90	0.93	>161 Alive	6
14	1.20	0.22	14	6
15	1.54	0.19	12	6
6	5.00	10.60	>203 Alive	9
16	2.77	3.20	8	9

TABLE 13. Mononuclear cell (MNC), total nucleated cell (TNC), and granulocyte/macrophage precursor cell (CFU-GM) activity per kg of body weight transfused in continuous flow centrifugation leukapheresis (CFCL) fractions to lethally irradiated dogs. (Numbers are mean \pm S.D.)

TABLE 13

**GRANULOCYTIC PRECURSOR ACTIVITY IN CFCL FRACTIONS TRANSFUSED TO
LETHALLY IRRADIATED DOGS**

Fraction Transfused	No. Dogs	CFU-GM/kg ($\times 10^{-4}$)	MNC/kg ($\times 10^{-8}$)	TNC/kg ($\times 10^{-8}$)	CFU-GM Activity per 10^5 MNC	CFU-GM Activity in Fractions (%)
CFCL	2	1.82	5.05	5.20	7.62	100
6.0 Gy		0.40	1.20	1.30	1.70	—
CFCL/Dex. Std.	2	0.89	3.45	4.00	5.05	100
6.0 Gy		0.06	2.19	2.55	0.24	—
CFCL	1	10.60	5.00	5.70	16.40	100
9.0 Gy		—	—	—	—	—

FIGURE 18. Peripheral blood leukocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates containing peripheral blood stem cells.

WHITE BLOOD CELL RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL CONCENTRATES

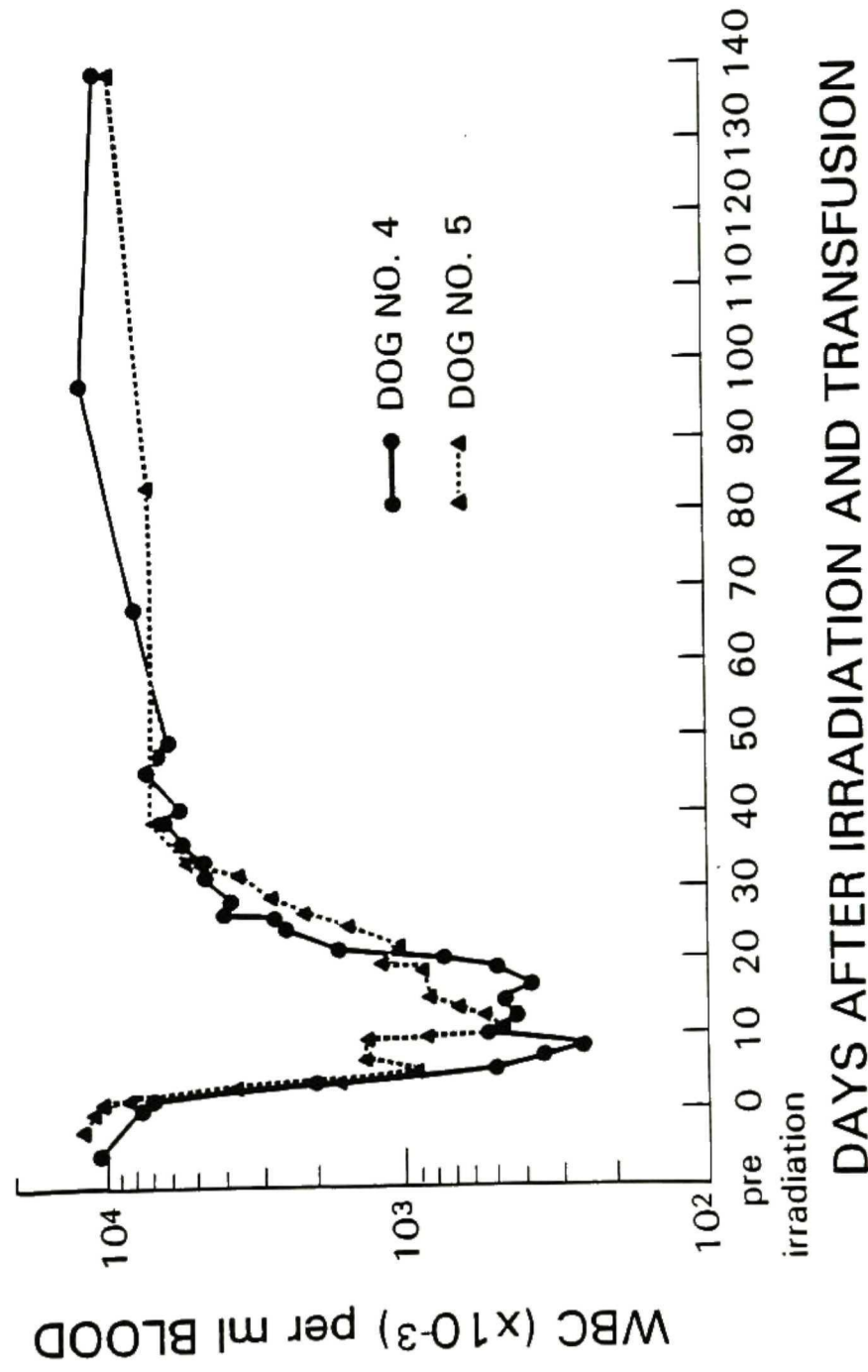


Figure 18

FIGURE 19. Peripheral blood erythrocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates containing peripheral blood stem cells.

RED BLOOD CELL RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL CONCENTRATES

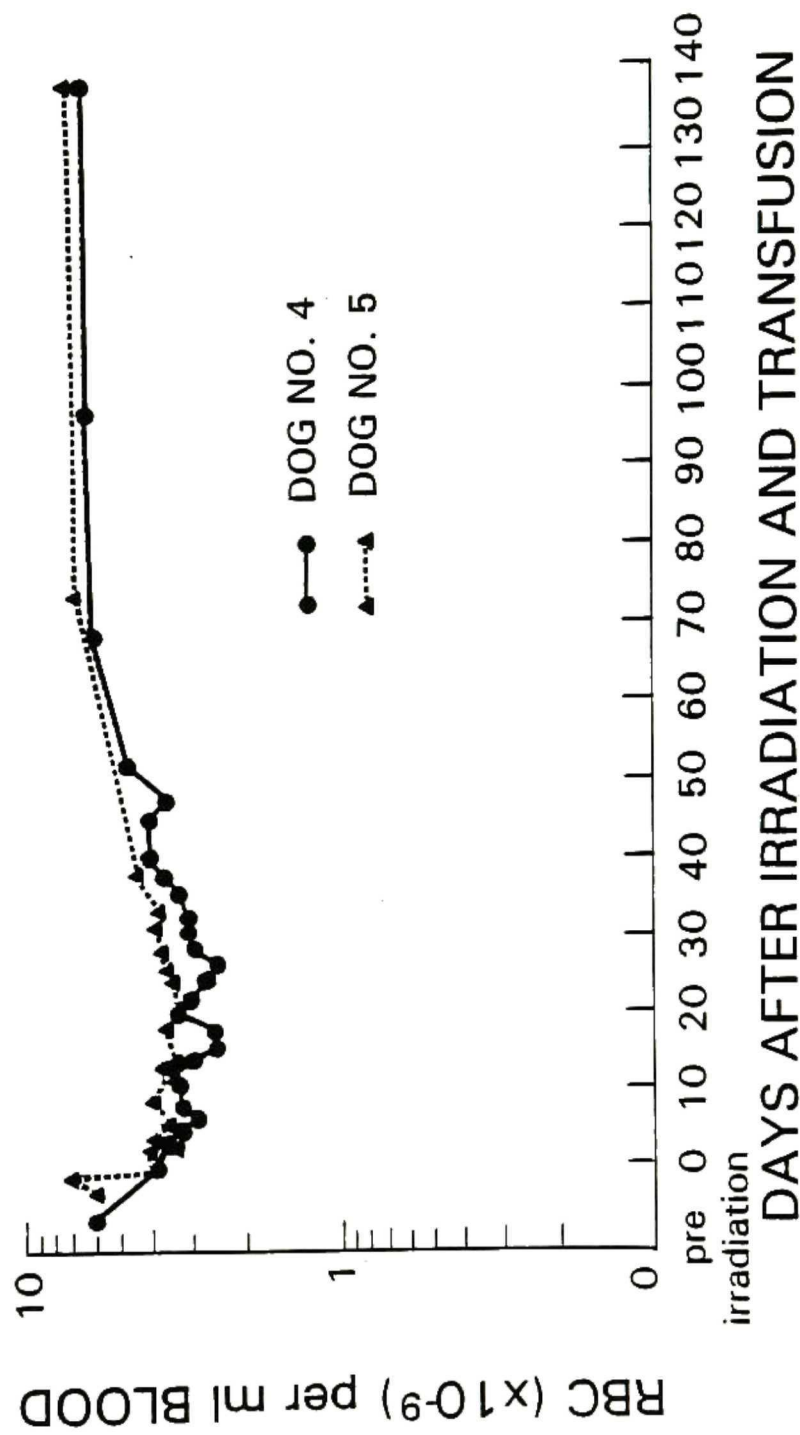


Figure 19

FIGURE 20. Peripheral blood platelet recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates containing peripheral blood hematopoietic stem cells.

PLATELET RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL CONCENTRATES

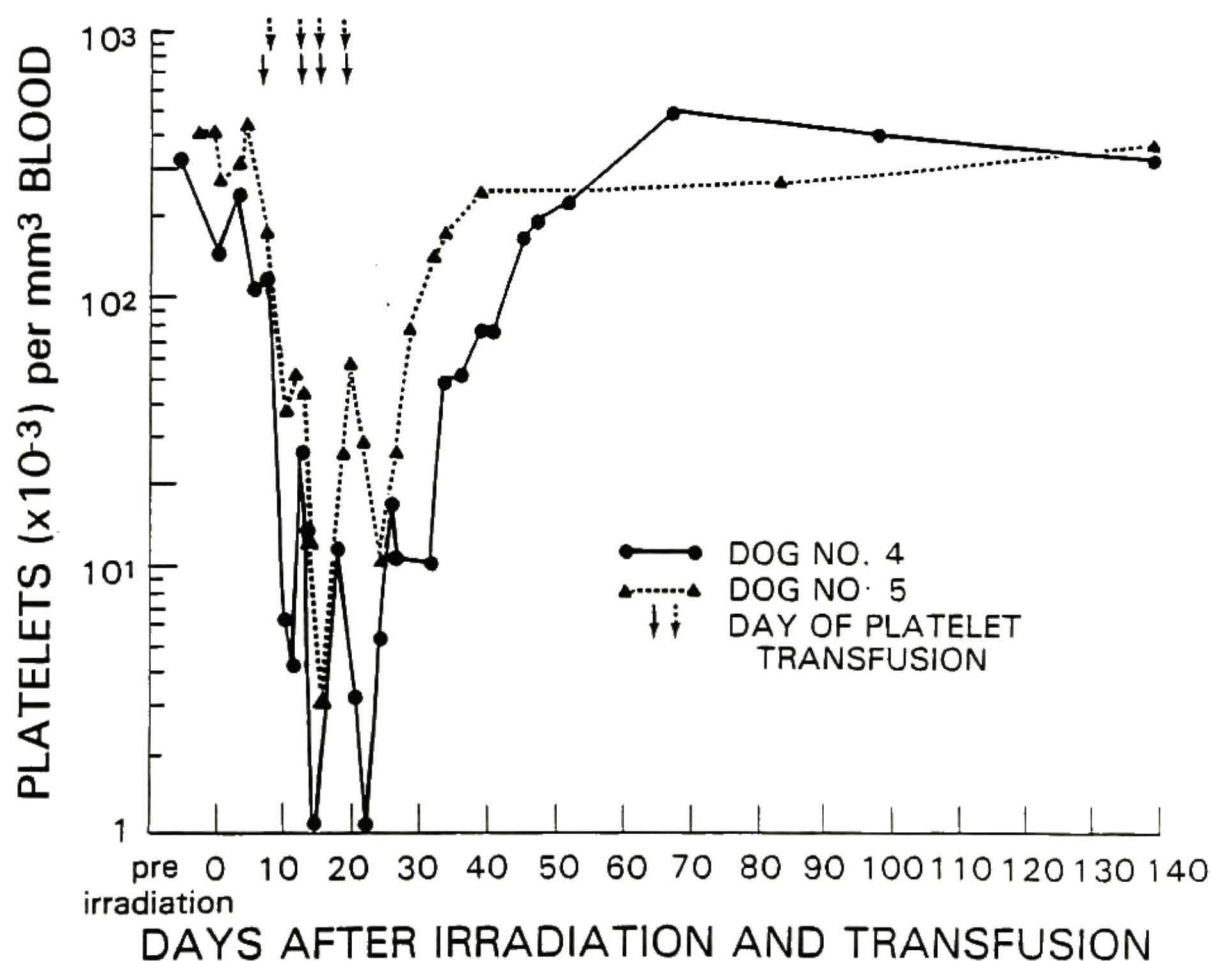


Figure 20

death syndrome.

Two dogs were irradiated with 9.0 Gy to test the upper limits of the established protocol's efficiency to mobilize and recover stem cells from peripheral blood. Dog No. 6 received a CFCL concentrate with a high number of MNC and an excellent CFU-GM activity (Table 12). Complete hematopoietic recovery was achieved by dog No. 6 within 58 days of irradiation and transfusion (Figure 21). Dog No. 16 (Table 12) received a CFCL concentrate of average MNC content and high CFU-GM activity (3.20×10^4 CFU-GM/kg). In spite of the fact that the CFU-GM activity was greater than the activity in concentrates received by four 6.0 Gy survival dogs, dog No. 16 did not survive beyond eight days after irradiation. These data were highly indicative of greater damage to the hematopoietic microenvironment (unpublished data) and greater irradiation induced complications to the animal as a whole. Necropsy reports indicated a death symptomatic of gastrointestinal death syndrome, as well as, hematopoietic death.

Erythrocyte-Depleted CFCL Concentrate Transfused Dogs

Prior to CCE fractionation it was necessary to remove the red blood cells. Thus, to determine whether sufficient stem cell activity remained after erythrocyte removal, three dogs were transfused with concentrates following dextran or ficoll-hypaque sedimentation. Dog No. 15 (Table 12) received an autologous transfusion of ficoll-hypaque sedimented cells with poor MNC and CFU-GM recovery. Again, cellular levels were insufficient for marrow engraftment. Dog No. 15 subsequently died 12 days later.

Dogs No. 7 and 8 received transfusions following dextran sedimentation to remove the erythrocytes from the CFCL concentrates. Dog No. 7, despite receiving greater than two and one half times more MNC per kg of body weight

FIGURE 21. The monitoring of the hematopoietic stem cell compartment recovery by following the peripheral blood erythrocyte, leukocyte and platelet recovery of the lethally irradiated dog exposed to 9.0 Gy mid-line tissue dose of gamma photons and transfused with a continuous flow centrifugation leukapheresis (CFCL) concentrate containing peripheral blood hematopoietic stem cells.

HEMATOPOIETIC RECOVERY OF DOG LETHALLY IRRADIATED WITH 9.0 Gy GAMMA AND TRANSFUSED WITH CFCL FRACTION

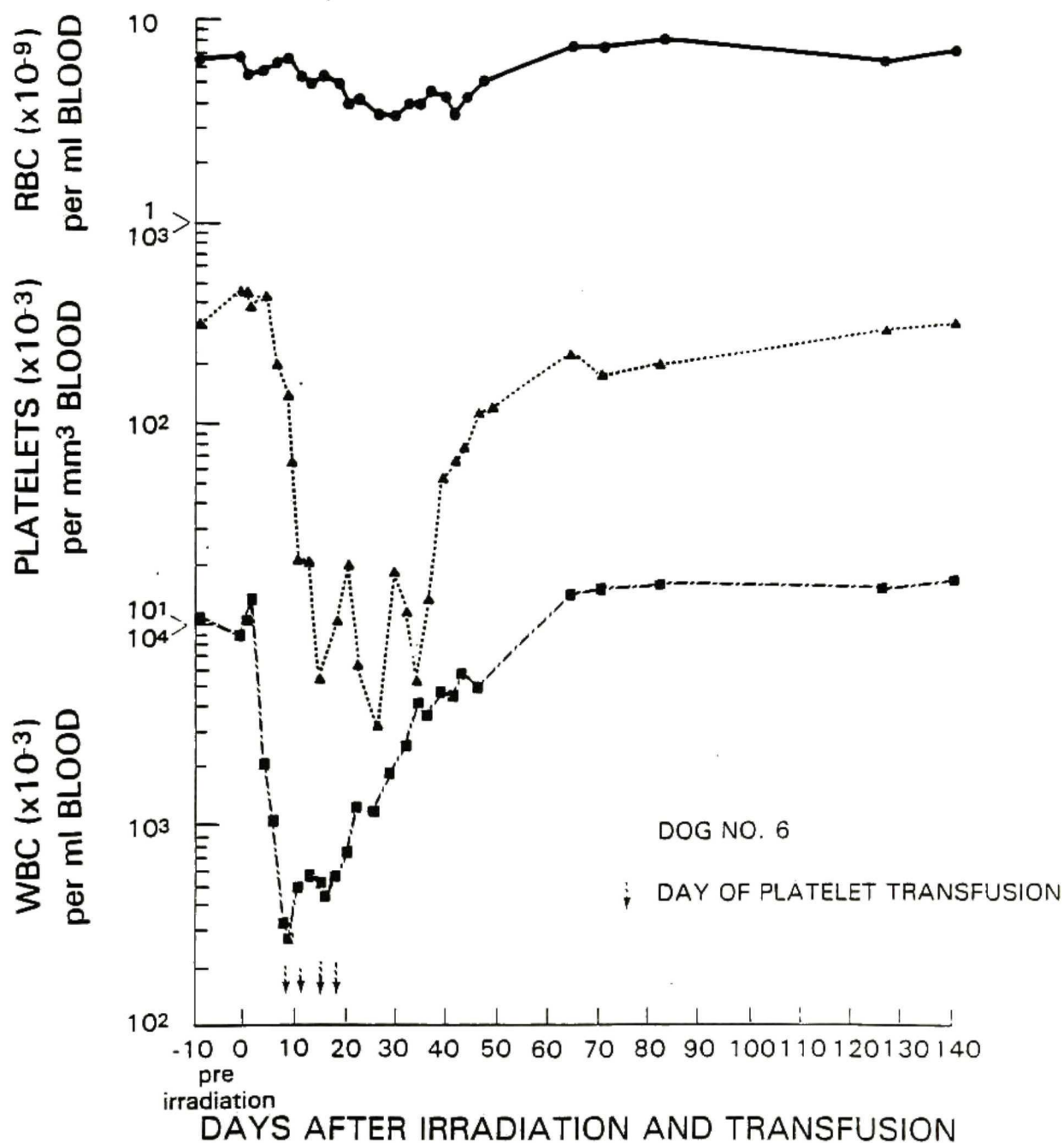


Figure 21

than dog No. 8, experienced a longer delay in complete hematopoietic recovery (Figures 22-24). The CFU-GM activity, however, of dog No. 7 was lower than that of dog No. 8. Complete hematopoietic recovery was evident in dog No. 8 60-days following irradiation and transfusion. Dog No. 7 did not achieve complete bone marrow reconstitution until 97 days after irradiation, almost five weeks later. Table 13 presents the mean cellular values of the concentrates given to dogs No. 7 and 8.

Figure 25 presents the rectal temperature records of irradiated dogs of four groups. Normal rectal temperatures of dogs as determined by us and others (Norris et al., 1968) is 38.6°C , however, temperatures as high as 39.3°C , related to excitement and handling procedures, are not uncommon. Therefore, for these experiments, temperatures of 40.0°C or greater, were arbitrarily considered of clinical significance. Basically, the onset of significantly elevated temperatures reflected the periods in which peripheral blood leukocyte counts were less than 1000 per mm^3 of blood. Of particular interest, in these studies, were the rectal temperatures manifested by recipient dogs of fraction I transfusions (Figure 25B). There was a lack of significant temperature elevations prior to day 18, unlike the other groups.

Dog Stem Cell Transfusions Following CCE Fractionation

Once it was established that following the leukopheresis and dextran sedimentation, sufficient stem cell activity had been recovered to achieve complete hematopoietic reconstitution of autologously transfused-lethally irradiated dogs, an in vivo assay for the confirmation of the presence of pluripotent stem cells was now practicable.

FIGURE 22. Peripheral blood erythrocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates following dextran sedimentation for removal of erythrocytes.

ERYTHROCYTE RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL/DEXTRAN SED. FRACTIONS

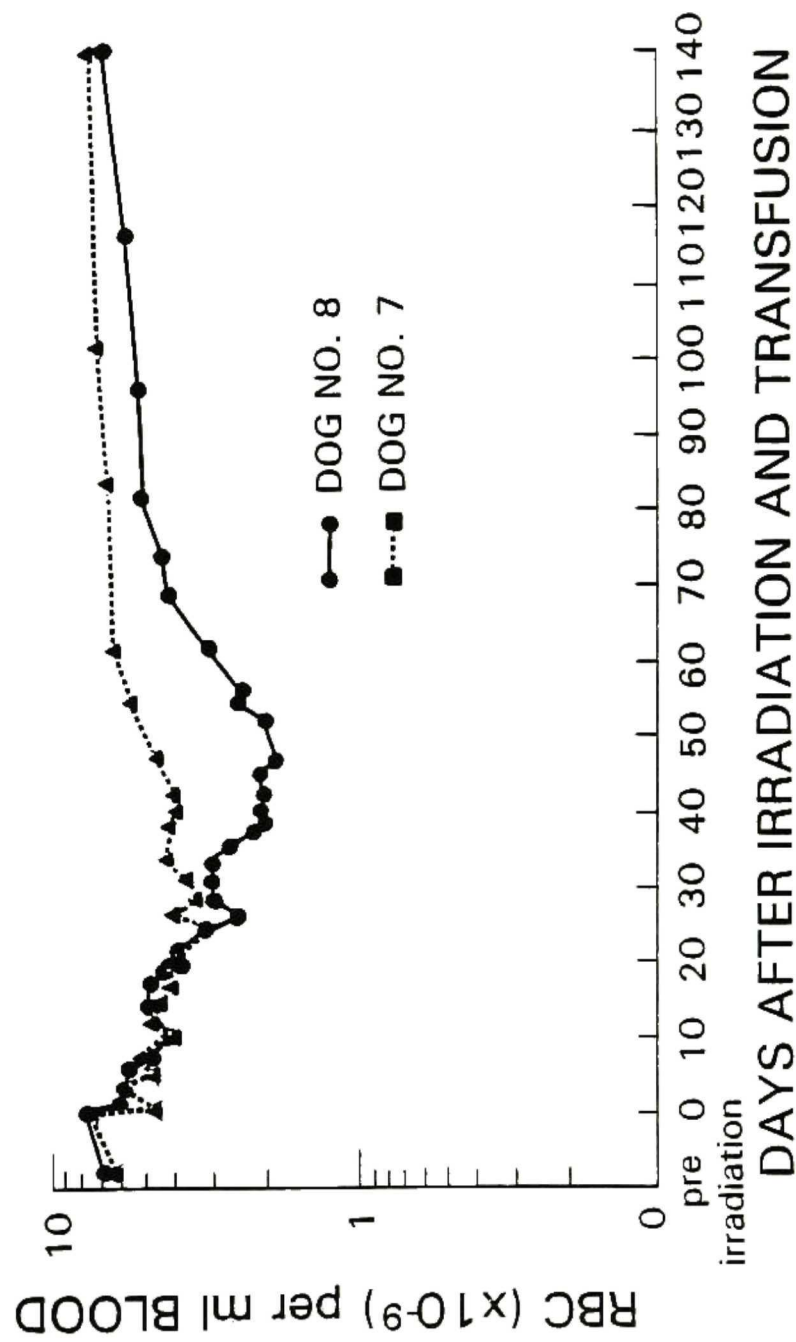


Figure 22

FIGURE 23. Peripheral blood leukocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates following dextran sedimentation for removal of erythrocytes.

WHITE BLOOD CELL RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL/DEXTRAN SED. FRACTIONS

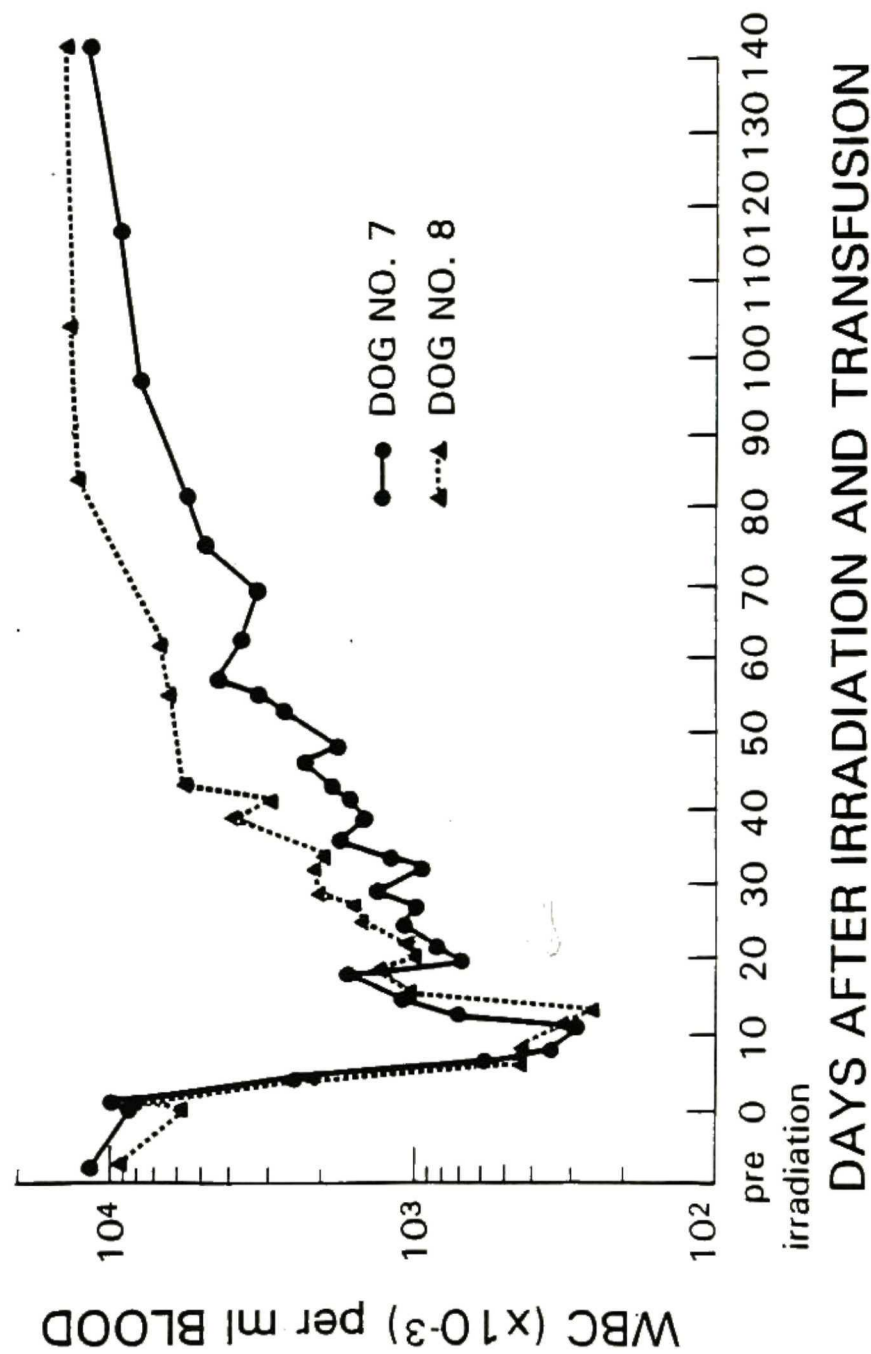


Figure 23

FIGURE 24. Peripheral blood platelet recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with continuous flow centrifugation leukapheresis (CFCL) concentrates following dextran sedimentation for removal of erythrocytes.

PLATELET RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH CFCL/DEXTRAN SED.FRACTIONS

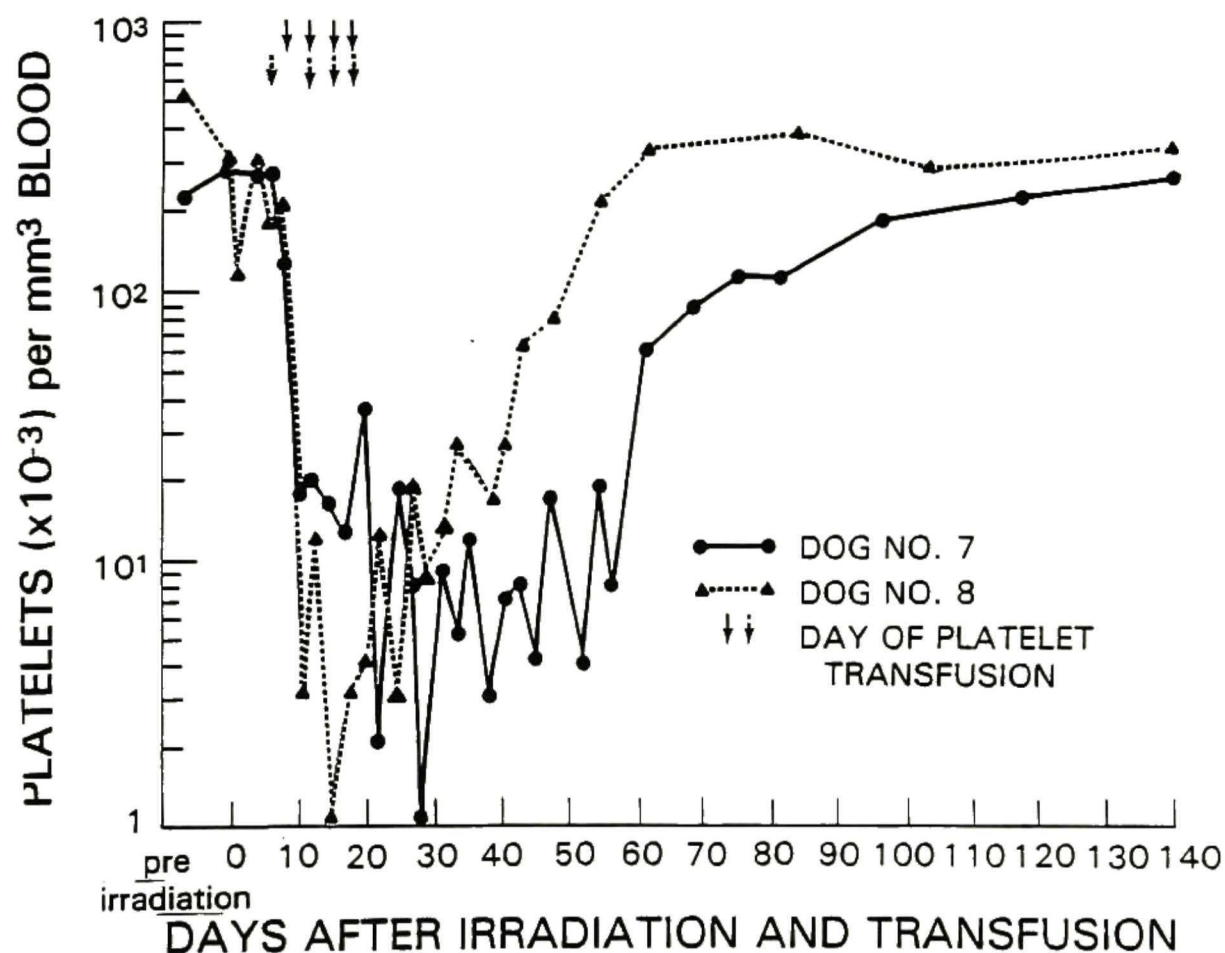


Figure 24

FIGURE 25. Rectal temperature record of four groups of lethally irradiated dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons.

RECTAL TEMPERATURE OF LETHALLY IRRADIATED DOGS WITH 6.0 Gy GAMMA MIDLINE DOSE

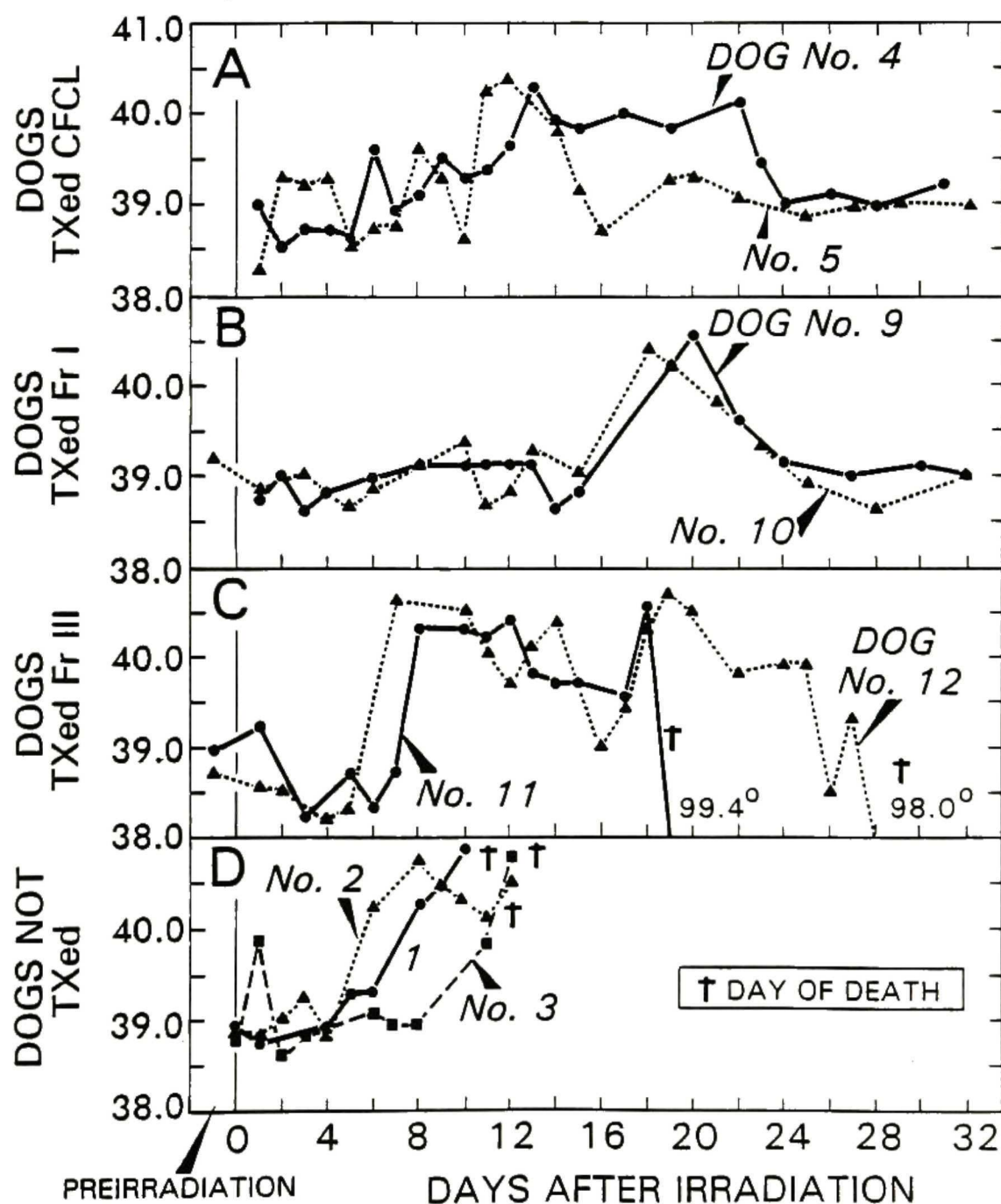


Figure 25

Two groups of dogs were transfused with either fraction I or fraction III, composed of CCE fractions as indicated on Figure 14. The committed stem cell activities, as well as morphological studies, of the transfused fractions were measured. Table 14 presents the mean CFU-GM activity of the two fraction I's and two fraction III's transfused. Fraction III, although comprised of an order of magnitude fewer MNC per kg of body weight, contained 27-fold greater CFU-GM/kg and 428-fold greater concentration of CFU-GM per 10^5 MNC. Fraction I contained a mean of only 3.8% of the total CFU-GM present in the elutriated fractions. Fraction III, on the other hand, contained a mean of 95.4% of all CFU-GM recovered in those elutriated fractions. The M-CFC data from two of these fractions, one fraction I and one fraction III, are presented on Table 10. Fraction I contained a mean of 1.2% M-CFC activity and fraction III contained a mean of 91.3% of the M-CFC activity. Four of the eight CFU-F experiment points used in Table 11 were derived from the fractions transfused in this study. As Table 11 indicates, fraction I had a mean of 1.9% of the total CFU-F activity and fraction III demonstrated a mean of 92.9% of the total CFU-F content in all fractions. The fractionation of the committed stem cell populations between fractions I and III was quite significant.

Survival data of the postirradiated fraction I recipients are depicted in Figures 26-28. Complete hematopoietic reconstitution was realized by dogs Nos. 9 and 10 within 42 days after irradiation and transfusion. In comparing recovery data of fraction I recipients with CFCL concentrate recipients (dogs No. 4 and 5, Figures 18-20), hematopoietic recoveries were quite similar. The major exception, in this comparison was in the leukocyte recovery profile. As shown in Figure 27, the peripheral blood white cell count never dropped below 800 leukocytes per mm^3 . However, examination of blood smears showed differentials of less than 30% circulating granulocytic cell types. Granulocyte counts,

TABLE 14. Mononuclear cell (MNC), total nucleated cell (TNC), and granulocyte/macrophage precursor cell (CFU-GM) activity per kg of body weight transfused to lethally irradiated dogs, following leukapheresis and elutriation (CFCL/CCE), as fraction I and fraction III. (All numbers are mean \pm S.D.)

TABLE 14

**GRANULOCYTIC PRECURSOR ACTIVITY IN CFCL/CCE FRACTIONS TRANSFUSED
TO LETHALLY IRRADIATED DOGS**

Fraction Transfused	No. Dogs	CFU-GM/kg ($\times 10^{-4}$)	MNC/kg ($\times 10^{-8}$)	TNC/kg ($\times 10^{-8}$)	CFU-GM Activity per 10^5 MNC	CFU-GM Activity in Fractions (%)
Fr. I	2	0.07	3.10	3.10	0.25	3.80
		0.01	1.60	1.60	0.21	0.70
Fr. III	2	1.90	0.31	2.40	107.00	95.40
		0.42	0.10	0.50	32.00	3.60

FIGURE 26.

Peripheral blood erythrocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation (CCE) fraction I containing less than four percent of all committed stem cell activity measured.

RED BLOOD CELL RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION I

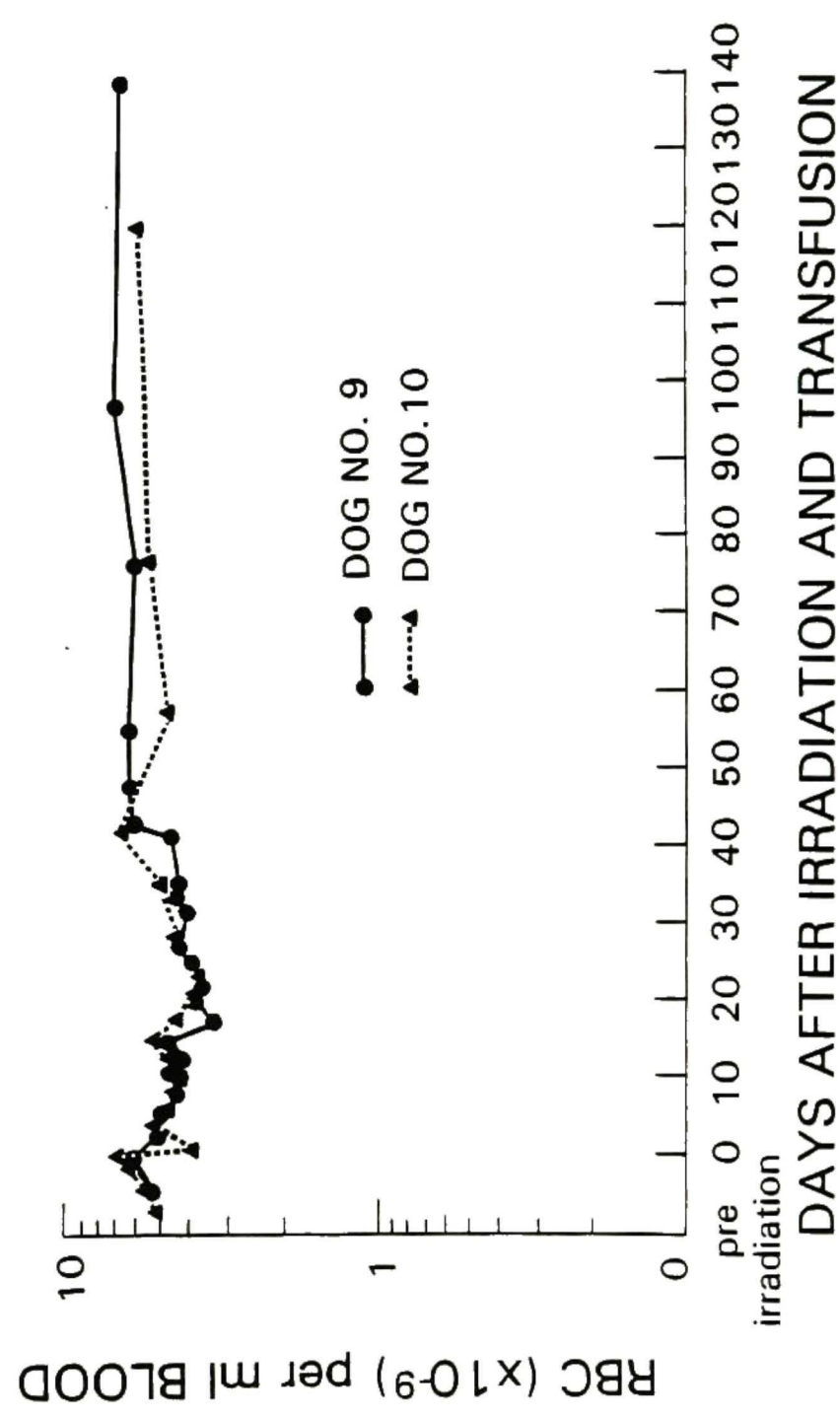


Figure 26

FIGURE 27. Peripheral blood leukocyte recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation (CCE) fraction I containing less than four percent of all committed stem cell activity measured.

WHITE CELL RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION I

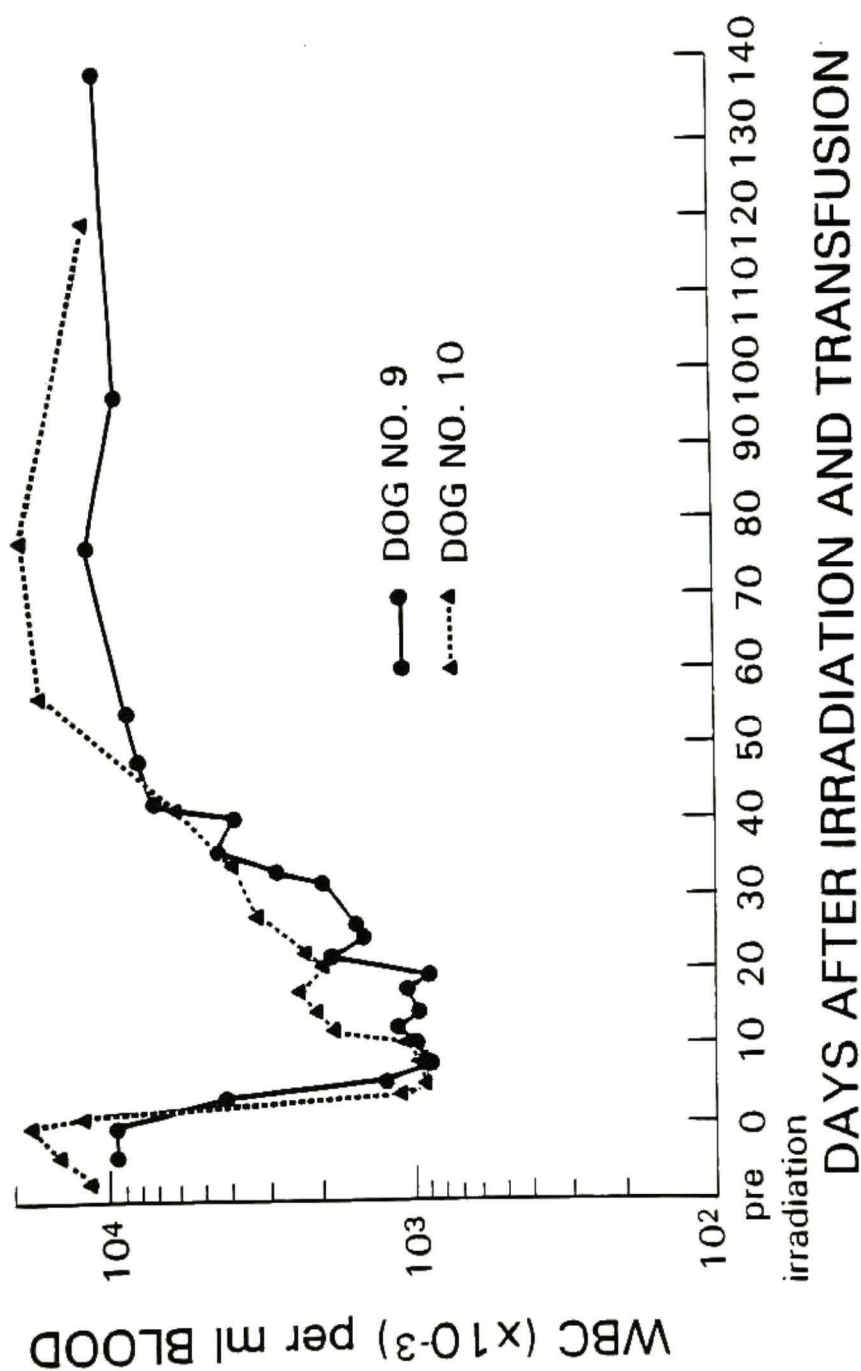


Figure 27

FIGURE 28. Peripheral blood platelet recovery of dogs exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation (CCE) fraction I containing less than four percent of all committed stem cell activity measured.

PLATELET RECOVERY OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION I

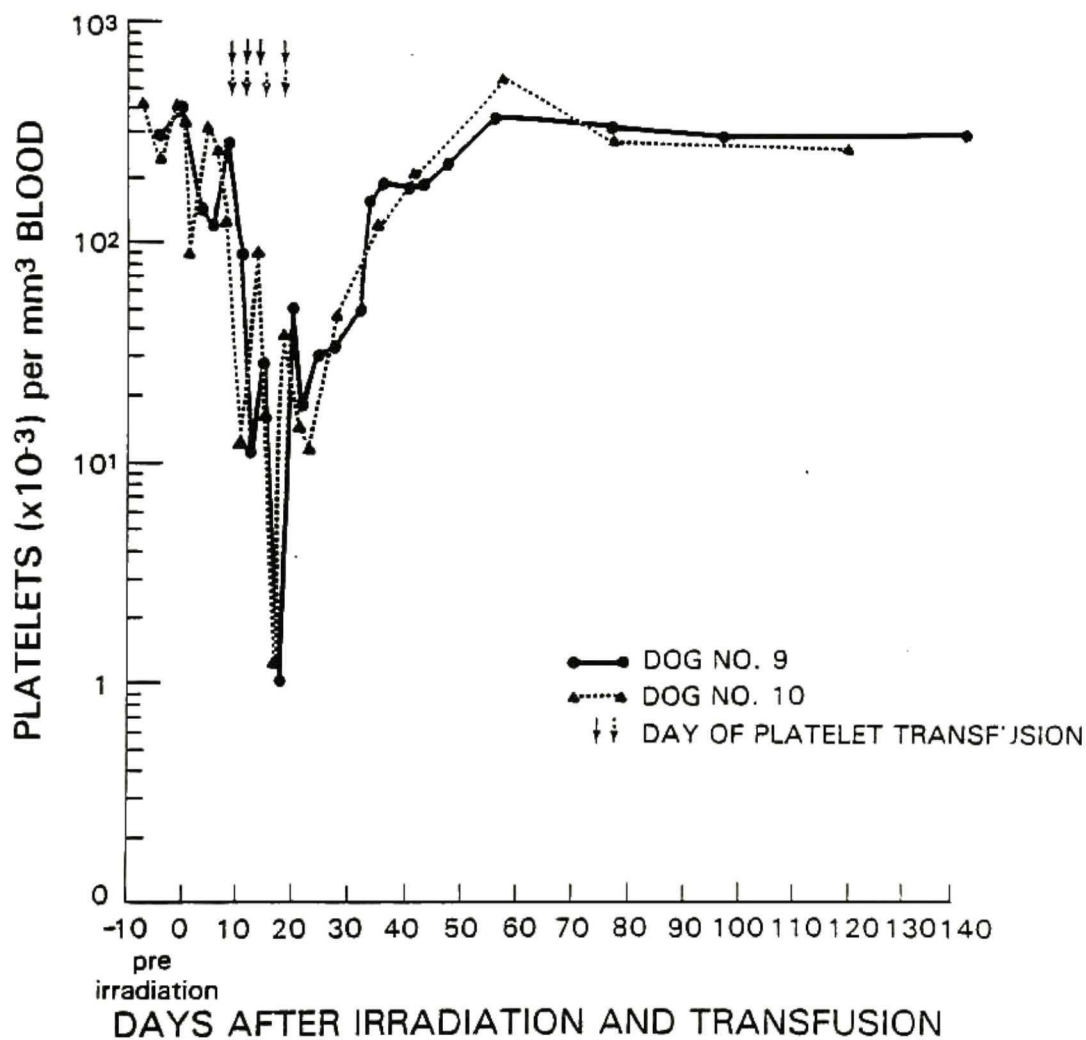


Figure 28

therefore, were approximately 225 per mm^3 of blood. The percent lymphocytes in these dogs, as determined by differentials, was higher during the neutropenic phase than that found in other groups of transfused dogs.

Post irradiation-hematopoietic recovery data of fraction III recipients are presented in Figures 29-31. The survival time of dogs Nos. 11 and 12 was significantly longer than that of the negative control dogs (Nos. 1-3). Dog No. 11 died 19 days after irradiation, and dog No. 12 died 29 days after irradiation. Figure 30 illustrates signs of hematopoietic activity prior to their deaths. Dogs Nos. 11 and 12, unlike the negative control dogs (Figure 16), showed a sustained elevation in leukocyte counts after the leukocyte nadir. Dog No. 11 reached the neutropenic nadir on day 10 and thereafter showed consistent WBC count increases for the next 16 days. Dog No. 12 reached its neutropenic nadir on day 8 and thereafter continued to produce leukocytes for the next 7 days and maintained a 1000 count per mm^3 blood for a minimum of two days. This data was suggestive of committed stem cell compartmental activity with no replenishment from the pluripotent stem cell compartment (Figure 1). Further evidence of hematopoietic activity in dogs Nos. 11 and 12 was derived from differential smears illustrating a marked shift to the left, i.e., the appearance of immature hematopoietic cells in the peripheral blood. Although, erythroid stem cell culture assays did not detect erythroid activity in any fraction, including fraction III, their existence was recognized by the presence of nucleated RBC in the peripheral blood of these hematopoietic recovering dogs.

Hematopoietic recovery data of a fraction III recipient is illustrated in Figure 32. Dog No. 13 was the recipient of a fraction III transfusion containing a significantly high percentage of lymphocytes or lymphocyte resembling cells (68%). The abnormal distribution of mononuclear cells indicated an unsuccessful fractionation. Committed stem cell culture assay data later strengthened this

FIGURE 29.

Peripheral blood erythrocyte counts of beagles exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation (CCE) fraction III containing greater than 95% of all committed stem cell activity measured.

RED BLOOD CELL COUNT OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION III

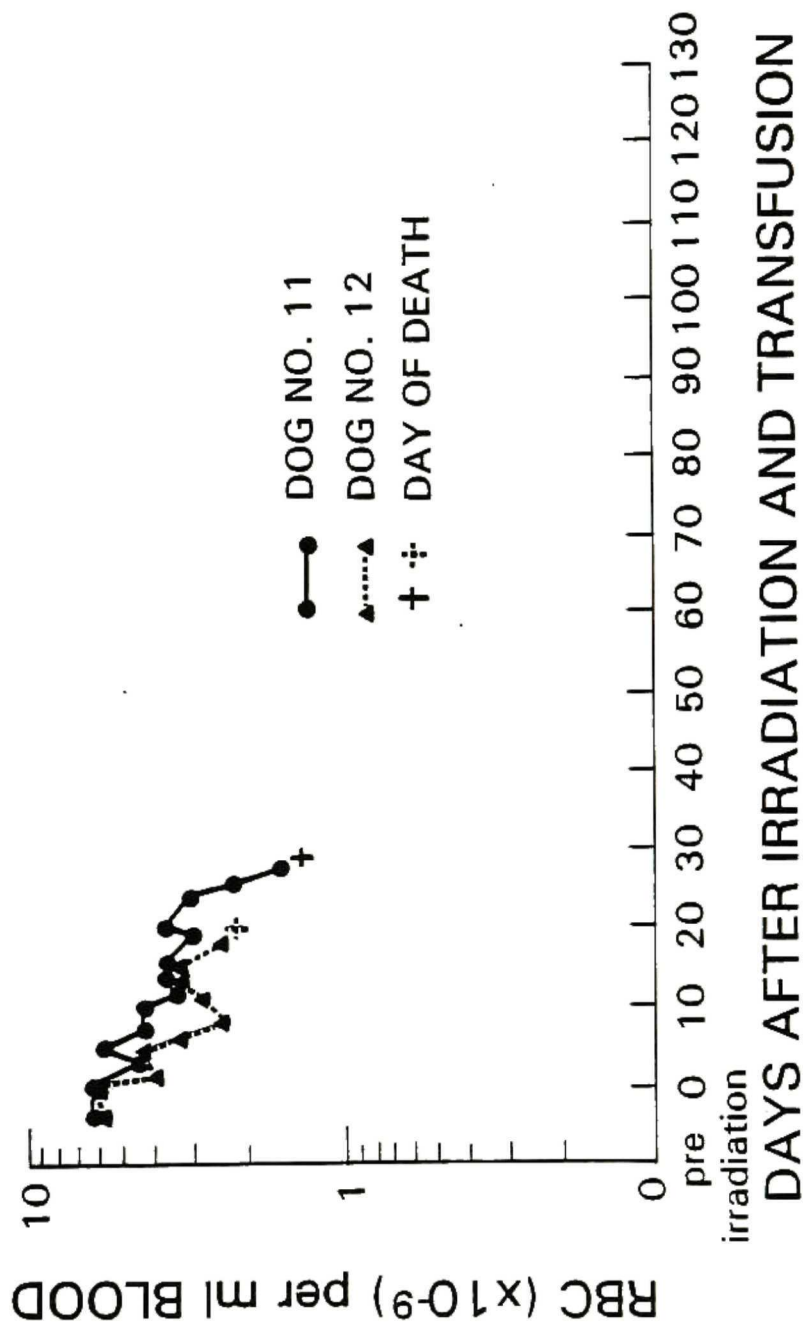


Figure 29

FIGURE 30.

Peripheral blood leukocyte counts of beagles exposed to 6.0 Gy mid-line tissue dose of photons and transfused with counterflow centrifugation elutriation (CCE) fraction III containing greater than 95% of all committed stem cell activity measured. A significant recovery in leukocyte counts was noted in fraction III recipient dogs as compared to control dogs after the nadir was reached ($p = 0.025$).

WHITE BLOOD CELL COUNT OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION III

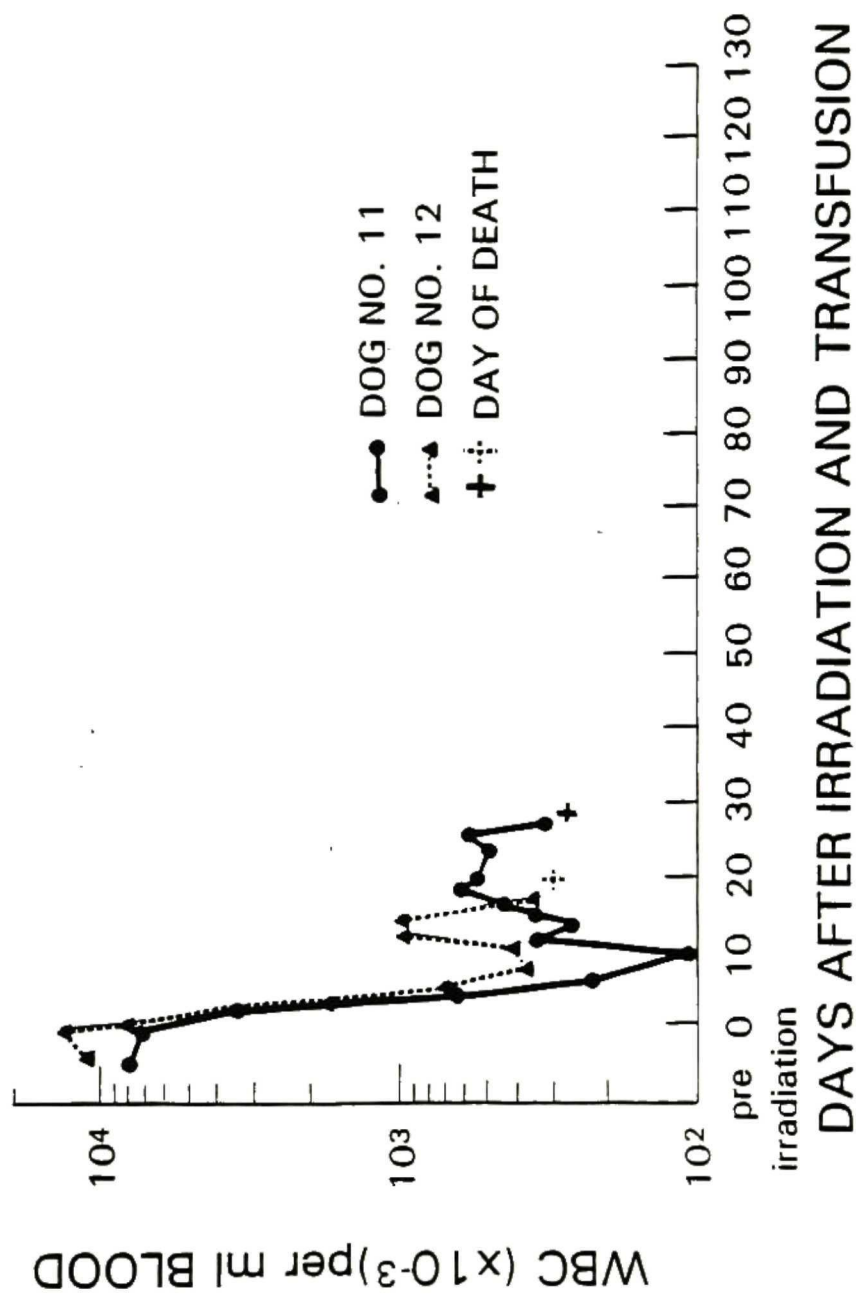


Figure 30

FIGURE 31. Peripheral blood platelet counts of beagles exposed to 6.0 Gy mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation (CCE) fraction III containing greater than 95% of all committed stem cell activity measured.

PLATELET COUNT OF LETHALLY IRRADIATED DOGS TRANSFUSED WITH FRACTION III

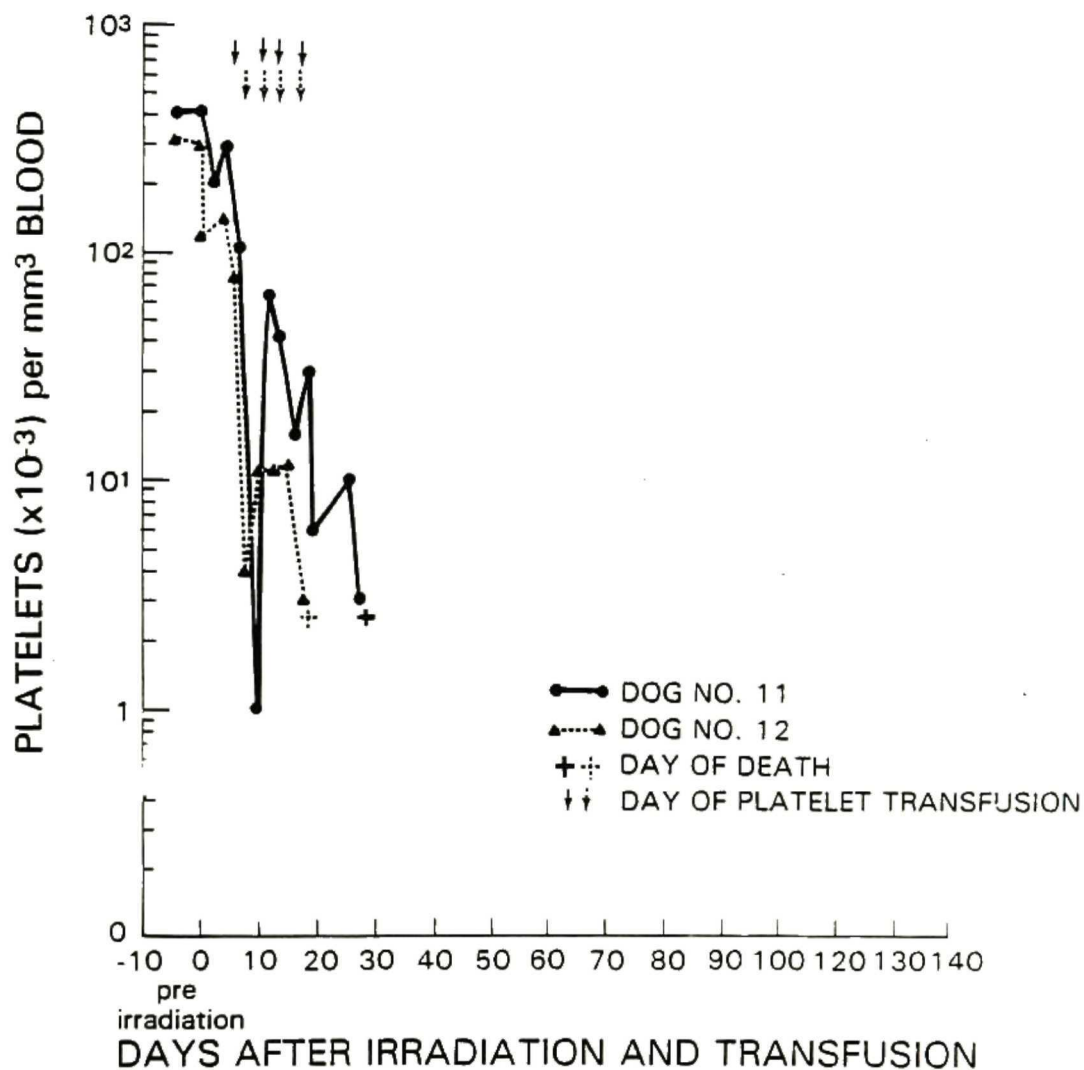


Figure 31

FIGURE 32. The monitoring of the hematopoietic stem cell compartment recovery by following the peripheral blood erythrocyte, leukocyte, and platelet recovery of a lethally irradiated dog exposed to 6.0 mid-line tissue dose of gamma photons and transfused with counterflow centrifugation elutriation fraction III containing a high percentage of immune competent lymphocytes (68%).

HEMATOPOIETIC RECOVERY OF LETHALLY IRRADIATED DOG TRANSFUSED WITH FRACTION III CONTAINING HIGH NUMBERS OF LYMPHOCYTES [68%]

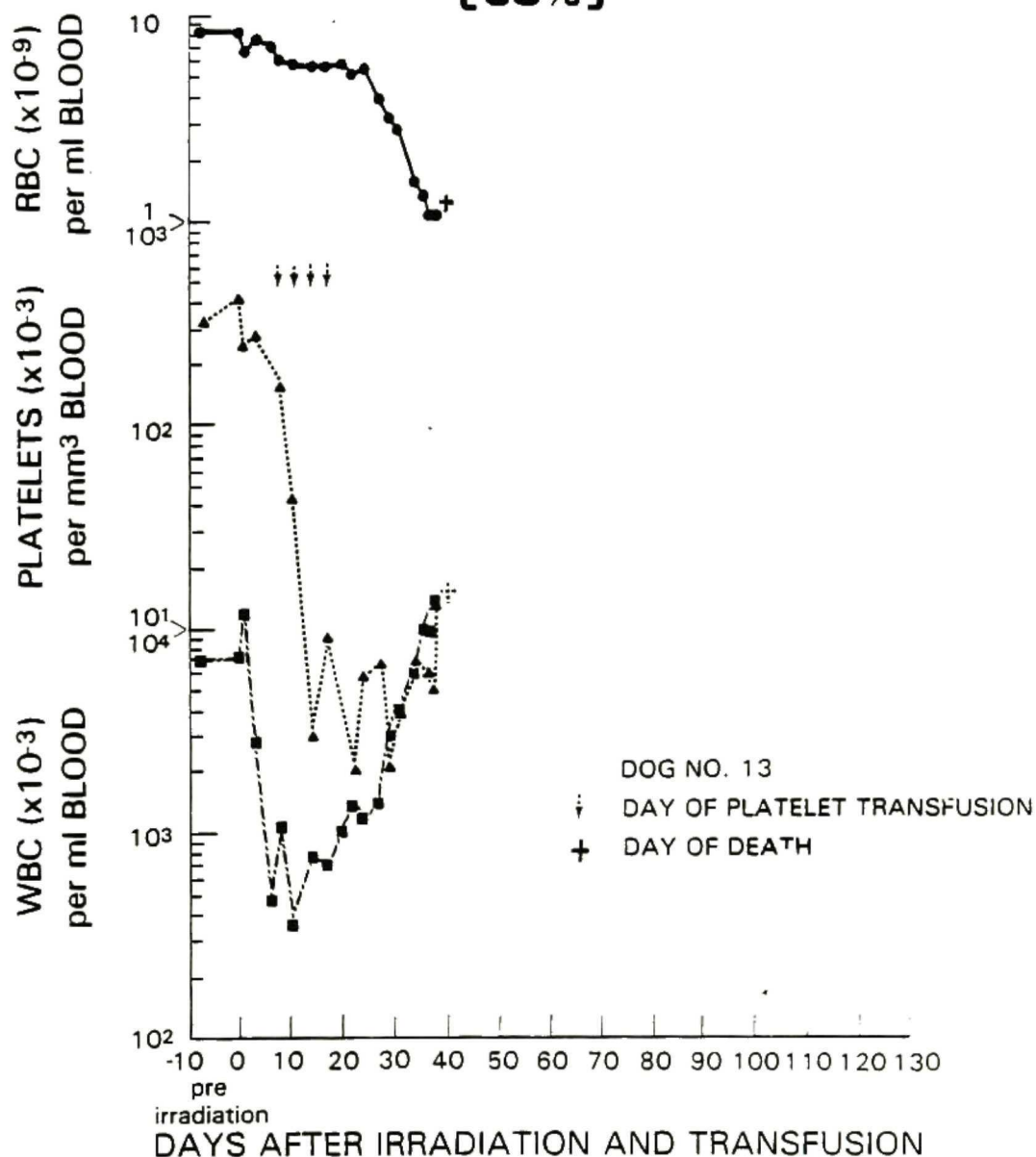


Figure 32

contention, as little separation had occurred. Further evidence of an unsuccessful distribution is presented in Figure 32. A return to normal, if not an elevation beyond normal in leukocytes, was seen in dog No. 13. Signs of platelet recovery were also present. However, as depicted, there was no erythrocyte recovery. Continuous hemorrhaging, beginning on day 24, due to intussusception (necropsy findings) and loss of appetite, lead to the dog's death on day 40 following irradiation. Peripheral blood smears, however, showed 19-34 nucleated erythrocytes per 100 total nucleated leukocytes in the circulation, starting at day 29 and continuing till death. This data was strongly suggestive of the presence and establishment of the pluripotent stem cell. The results of the autologous transfusions of post elutriation fractions I and III, clearly demonstrate the clean physical separation of committed stem cells from the pluripotent stem cells.

DISCUSSION

I. EVIDENCE FOR THE EXISTENCE OF A BONE MARROW-BLOOD BARRIER IN HUMANS AND CANINES

The spontaneous presence of homogeneous populations of committed stem cells in the peripheral circulation of humans and canines has been clearly demonstrated in this investigation. These findings appear to provide convincing evidence for the existence of a bone marrow-blood barrier that permits the passage of specific subpopulations of committed stem cells into the peripheral blood. The numbers of these circulating stem cells can be significantly increased in dogs by the administration of dextran sulfate, without altering the elutriation profiles' depiction of homogeneous stem cell populations. These data strongly indicate that the spontaneously circulating subpopulations are identical with the mobilized subpopulations of committed stem cells found in the blood of dogs. Therefore, the selectivity of the bone marrow-blood barrier was not disturbed by dextran sulfate despite its enhancement of stem cell passage across the barrier. In comparing the findings of this investigation with those of bone marrow (Jemionek et al., 1982a and 1982b) it was concluded that the CFU-GM circulating in blood had a mean cell volume which was greater than that of CFU-GM found in the bone marrow.

Gerhartz and Flidner (1980) and Gerhartz et al. (1982) employing velocity sedimentation and tritiated thymidine induced cell death techniques, have provided evidence that circulating CFU-GM are derived from a specific subpopulation within the bone marrow, the so called 'mobilizable CFU-GM fraction'. These cells were characterized by their smaller size and lower S-phase fraction than CFU-GM found in the bone marrow. They also reported that both the CFU-GM that accumulated in the blood within 1-3 hours after dextran sulfate

administration and the spontaneously circulating CFU-GM are fairly homogeneous with respect to their size and their cytokinetic state, and therefore most likely belong to an identical cell population. On the other hand, the CFU-GM in the bone marrow are more heterogeneous in size and are on the average larger, with slightly different cytokinetics. From these studies the authors suggested the existence of a bone marrow-blood barrier that prevents larger bone marrow CFU-GM from entering the peripheral blood under normal conditions. They also suggested that dextran sulfate mobilization does not alter the selectivity of the bone marrow-blood barrier.

Our findings, with respect to the sharp separation of CFU-GM from the vast majority of the lymphocytes and the narrow CFU-GM fractionation profiles (Figures 12, 13, and 14), together with the evidence obtained from Tables 3, 6, and 9, suggest a homogeneous population of CFU-GM present in peripheral blood of both canine and human. In contrast, previous studies in our laboratory on the CCE fractionation of mammalian bone marrow (Jemionek et al., 1982a) reported heterogeneous profiles for human and canine CFU-GM bone marrow populations. Jemionek et al. (1982a) have observed two separate CFU-GM peaks in canine bone marrow CCE fractionation profiles. Approximately one-third of the total CFU-GM was associated with the early nucleated cell fraction and the other two-thirds of the CFU-GM were associated with the second nucleated cell population. Human bone marrow fractionation studies, also resulted in a heterogeneous CFU-GM population. The CFU-GM profile indicated a small population of CFU-GM associated with the initial nucleated peak. Less than 15% of the total CFU-GM recovered were associated with this peak. However, unique to the human bone marrow were two major peaks of CFU-GM activity occurring in the latter fractions. This two peak profile occurred in all human studies. Mouse and monkey bone marrow fractionations also showed extensive heterogeneity in the CFU-GM

population profiles. Williams and Moore (1973) also showed significant heterogeneity in the monkey CFU-GM bone marrow population and Bol et al. (1979) have observed the same heterogeneity in mouse bone marrow.

These findings clearly demonstrate that the homogeneity of the CFU-GM population found in peripheral blood was quite in contrast to the heterogeneity of the CFU-GM population found in the bone marrow. These data are indicative of the presence of a normal committed stem cell selection process occurring during the passage of cells into the peripheral circulation from the bone marrow. The CFU-GM population selection for migration did not only occur in dogs but also in humans. Thus our observations in the dog strongly support Gerhartz and Flidner's (1980) and Gerhartz's et al. (1982) contention of the presence of a selective bone marrow-blood barrier. However, our findings further suggest the presence of such a barrier in humans.

Of greater significance for this study were the new findings derived from the CCE fractionation of M-CFC. M-CFC fractionation data presented evidence of the spontaneous presence and mobilization, into peripheral blood, of homogeneous populations of macrophage progenitor cell as well. Although the M-CFC fractionation profiles (Figures 12 and 13) illustrate less homogeneous populations than CFU-GM, in actuality M-CFC were isolated in only a few fractions of each fractionation study. However, M-CFC frequently preceded CFU-GM exit from the separation chamber. In normalization of these data (see Materials and Methods) for graphic representation, the M-CFC profiles appear broader and thus less homogeneous. Further evidence of M-CFC peripheral blood homogeneity is obtained from Tables 4, 7, and 10. These data indicated a peripheral blood M-CFC population homogeneity, in both dog and human, very similar to that observed for CFU-GM populations. These findings thus show that the bone marrow-blood barrier postulated for both dog and human was not only

selective for CFU-GM but also for M-CFC.

This investigation afforded more new evidence for the postulated barrier in dogs. Results of the fractionation of stromal precursor cells (CFU-F) also suggest mobilization, into peripheral blood, of a homogeneous population of stromal stem cells (Table 11). Similar results were also observed in megakaryocytic progenitor (CFU-M) fractionation studies (data not shown). Thus, the data presented strongly indicate that the bone marrow-blood barrier is not only selective for uniform subpopulations of CFU-GM and M-CFC in dogs, but also selective for CFU-F and CFU-M.

The mobilization of the erythroid precursors, BFU-E and CFU-E was not observed in the peripheral circulation of the dog and was not assayed for in humans. Erickson and Torok-Storb (1981) have demonstrated that bursts (BFU-E) could be grown from peripheral blood mononuclear cells whereas colonies (CFU-E) could not. Their attempts to concentrate BFU-E by density centrifugation resulted in no growth in any density fraction. Burst formation was again observed when the fractions were recombined, suggesting an "obligatory auxillary cell may have been separated from BFU-E by density" centrifugation. These results may explain our failure to observe BFU-E in our studies. The inability to demonstrate the presence of CFU-E in dog peripheral blood, however, may indicate ineffective techniques for their growth in culture or, more interestingly, may suggest a selectivity of BFU-E by the bone marrow-blood barrier and not for CFU-E in dogs. In humans, again, only the BFU-E have been observed (Ogawa et al., 1977; Clarke et al., 1977) in the peripheral circulation, suggesting that the human bone marrow-blood barrier, as in dogs, permits only the passage of the more immature erythroid precursor which apparently does not differentiate into CFU-E in the circulation.

Our observations showed no variations in CCE fractionation profiles,

whether the CFU-GM or M-CFC peripheral blood populations were obtained from normal dogs, i.e., spontaneously circulating populations, or from dogs given dextran sulfate, i.e., mobilized populations. These data are supported by Gerhartz and Flidner's (1980) and Gerhartz's et al. (1982) contention that dextran sulfate does not change the selectivity of the bone marrow-blood barrier for stem cell subpopulations mobilized into peripheral blood despite influencing greater stem cell mobilization.

The structure and composition of the bone marrow-blood barrier is at this time conjectural and implies an intercellular movement of stem cell into the peripheral blood. However, other mechanisms of stem cell selectivity have been suggested. Van der Ham et al. (1977) and Vos et al. (1979) have suggested the complement system as a possible candidate for a cofactor involved in the rapid mobilization, within minutes, of CFU-S from the marrow into blood by bacterial lipopolysaccharides. Wilschut et al. (1977) provided evidence for a role of C3 in the mobilization phenomenon. The slower CFU-S and committed stem cell mobilization by agents such as dextran sulfate, and concanavalin A may be mediated by a different unknown mechanism. These mechanisms suggest that pores or channels may exist between the bone marrow and peripheral blood and that various chemical agents can selectively alter the rate of stem cell movement through these channels without altering the cell selectivity of the channel.

Although our findings support Gerhartz and Flidner's (1980) hypothesis of a bone marrow-blood barrier selectivity, our results do not support their observations that the circulating CFU-GM are smaller than granulopoietic precursors of the bone marrow. Our laboratory has observed (Jemionek et al., 1982b) that at least 25-40% of the dog bone marrow CFU-GM recovered in CCE fractionation studies were associated with the early exiting nucleated cell peak. These data were indicative of a more buoyant, i.e., smaller, less dense, cell. On

the other hand, our findings show that less than 6% of the dog peripheral blood CFU-GM exited with the early nucleated cell peak. Jemionek et al. (1982a) reported that approximately 85% of the human bone marrow CFU-GM was cells having cell volumes of $291-350 \text{ um}^3$. In these studies approximately 90% of the human peripheral blood CFU-GM were isolated with cells having a mean cell volume of 378 um^3 . These results indicate a peripheral blood population of CFU-GM, in humans and dogs, with a mean size larger than the mean size of the bone marrow CFU-GM. Giladi et al. (1974) and Micklem et al. (1975) have shown that circulating CFU-GM are more mature, with lower self-renewal capabilities than CFU-GM found in the bone marrow. These reports, in conjunction with the findings of Worton et al. (1969), Sutherland et al. (1971) and Jacobsen et al. (1979) indicating that CFU-GM of relatively smaller size and Jacobsen et al. (1979) indicating that CFU-GM of relatively smaller size have higher capacity for self-renewal than those of larger CFU-GM, support our findings.

Committed Stem Cell Concentration

Counterflow centrifugation elutriation, employing the POMEX technique, was demonstrated to be a useful tool in the concentration of committed and possibility pluripotent stem cells. Human CFU-GM and M-CFC activities per 10^5 lymphocytes were concentrated 50-fold over pre-elutriation values on the smaller separation chamber. Dog CFU-GM and M-CFC concentrations on the smaller chamber were even more dramatic. Stromal cell progenitor activity was also markedly increased along with that of CFU-GM and M-CFC utilizing the larger separation chamber.

Bone Marrow Mobilization

It was clearly demonstrated in these studies that the heparinoid polyanion dextran sulfate initiated the mobilization of the granulocyte/macrophage progenitor cell (CFU-GM) into the peripheral blood. Ross et al. (1977 and 1978), Nothdurft et al. (1982), and Gerhartz and Fliedner (1980) have all used dextran sulfate with molecular weights of either 10,000 or 40,000 prepared in their laboratories. The dextran sulfate used for our studies was of an 8,000 molecular weight of a commercial source, facilitating its accessibility.

Our twelve-fold CFU-GM mobilization results were comparable to those of Ross et al. (1977 and 1978) and Gerhartz and Fliedner (1980). The two-fold increase in circulating mononuclear cells has also been reported by Ross et al. (1977). These data strongly suggest that dextran sulfate may induce the selective release of CFU-GM from extravascular sites, most likely from bone marrow storage sites, in preference to mononuclear cells and polymorphonuclear leukocytes. There was ample evidence that along with CFU-GM, the macrophage committed stem cell (M-CFC) was also selectively released. The mobilization of the megakaryocytic progenitor cell, (CFU-M), as well as that of the stromal progenitor cell (CFU-F) was also observed. The erythroid assay for BFU-E and CFU-E quantitation did not indicate the presence of either erythroid precursor in the peripheral blood of dogs, in any of these studies.

The mobilization of the CFU-F was an unexpected event, however, since the CFU-F is a stromal stem cell, its mobilization, along with other stem cell types, should also be expected. Its mobilization was approximately two orders of magnitude lower than that of CFU-GM and M-CFC, suggesting that the fibroblastic precursor cell was more sessile or entrenched within the bone marrow,

fewer CFU-F were available for migration into the peripheral blood and/or that dextran sulfate did not selectively stimulate its mobilization.

The correlation between CFU-GM and M-CFC mobilization and dog survival postirradiation (Table 12) indicated that there was a direct relationship between CFU-GM and M-CFC activities and pluripotent stem cell activity in all leukapheresis concentrates. Furthermore, fraction I transfusions, resulting in complete hematopoietic recovery had corresponding fraction III's with high CFU-GM and M-CFC activities. These findings suggest that dextran sulfate influenced the mobilization of the pluripotent stem cell in association with committed stem cells.

Rickett et al. (1953) and Walton (1954) have shown lower molecular weight dextran sulfate to be non-toxic. Double dextran sulfate regimens used for greater mobilization prior to leukapheresis had no notable adverse effects on the animals.

Nothdurft et al. (1982) reported that the mobilization of CFU-GM from extravascular sites, in dogs using dextran sulfate, was depending on the number of CFU-GM present in the blood at the time of mobilization, i.e., higher initial spontaneously circulating stem cells levels in the blood prior to mobilization resulted in greater numbers of CFU-GM mobilized. Our results using 15 mg/kg dextran sulfate confirmed these observations. Our findings also supported those of Nothdurft et al. (1982), in respect to their observations of fluctuations in the absolute numbers of dextran sulfate-mobilized CFU-GM between individual normal dogs, as a factor of 3-4 between minimum and maximum levels. These fluctuations may be due to variations in the pool size, under normal conditions, of circulating and mobilizable CFU-GM as reported by Nothdurft and Flidner (1982) in dogs and by Barrett et al. (1979) and Grilli et al. (1980) in man.

Our mobilization data using 7.5 mg/kg dextran sulfate was similar to that reported by Ross et al. (1978) who used 10 mg/kg dextran sulfate. In both cases lower overall CFU-GM mobilization levels were achieved when compared to the administration of 15 mg/kg dextran sulfate. Nothdurft et al. (1982) also reported observing higher mobilization values with 20 mg/kg dextran sulfate infusions than with 15 mg/kg on normal dogs. Our findings with 30 mg/kg dextran sulfate administration suggest that there is an optimal concentration for maximal mobilization.

Peripheral Blood Stem Cell Procurement By Continuous Flow Centrifugation Leukapheresis

To obtain an equal number of mononuclear cells on the Aminco Celltrifuge, the leukapheresis procedure had to be used twice as long as compared with that of the IBM 2997 Blood Cell Separator. The stem cell mobilization with dextran sulfate also provided us with almost a nine-fold increase in the number of CFU-GM collected in approximately one half the time. The IBM 2997 was also more selective in harvesting mononuclear cells than the Celltrifuge or other leukapheresis instruments used for the collection of mononuclear cells and peripheral blood stem cells (Kovacs et al., 1978; Ross et al., 1977). Of significance to the simplification of these studies, is the fact that dogs leukapheresed on the IBM 2997 averaged half the weight of those leukapheresed on the Celltrifuge.

The successful leukapheresis of 8-13 kg beagles on the IBM 2997, demonstrates the benefit of the instrument's requisite for low extracorporeal volumes. The ability to utilize a smaller sized dog, in lieu of heavier beagles or foxhounds, made the overall management of this investigation more lucrative. Housing problems were markedly reduced, daily handling of dogs for clinical

observations and support were much facilitated, and above all, the total number of stem cells required per transfusion were fewer; therefore, allowing for shorter leukapheresis procedures. Consequently, the budgeting of time on days of mobilization, leukapheresis, irradiation, transfusion, followed by support of the animals, all on the same day, was made much more manageable.

Erythrocyte Removal From CFCL Concentrates Prior to CCE Fractionation

The removal of erythrocytes by the ficoll-hypaque sedimentation technique proved to be a deleterious approach to facilitate CCE fractionations. The loss of mononuclear cells and CFU-GM activity was acceptable for elutriation studies on the smaller separation chamber. However, these cell losses were too large to achieve successful bone marrow engraftments. Koerbling et al. (1977) and Ross et al. (1977) have also reported such losses with ficoll-hypaque. Because of these losses, dextran sedimentation was subsequently used for CFCL concentrate preparation prior to elutriation on the larger separation chamber. Dextran sedimentation resulted in low residual erythrocyte numbers and excellent mononuclear cell and CFU-GM recoveries.

II. PERIPHERAL BLOOD TRANSFUSIONS OF LETHALLY IRRADIATED DOGS TO ASCERTAIN THE PHYSICAL SEPARATION OF PLURIPOTENT AND COMMITTED STEM CELLS

For complete hematopoietic reconstitution of any bone marrow to occur, the presence of the pluripotent stem cell (CFU-S), or its equivalence, is essential. Thus our findings with respect to survival of lethally irradiated dogs receiving fraction I cells are very significant. These dogs achieved complete hematopoietic recovery within 42 days after irradiation and autologous transfusion. The rate of hematopoietic recovery was quite similar to that in dogs receiving unsedimented, unfractionated leukapheresis autologous concentrates, in spite of the fact that the fraction I dogs received less than four percent CFU-GM per kg of body weight than did the CFCL recipient dogs. These results indicate that despite the huge disparity in committed CFU-GM stem cell content, the pluripotent stem cell content was similar. The re-establishment of the lymphoid peripheral blood populations was strongly indicative of the presence and engraftment of the primitive pluripotent stem cell as well (Figure 1). Fraction III recipient dogs, on the other hand, were transfused with the same number of CFU-GM per kg of body weight as the leukapheresis concentrate recipient dogs and survived only a mean of 24 days. Hematopoietic recovery profiles of fraction III recipient dogs, were suggestive of committed stem cell compartmental activity with no replenishment from the pluripotent stem cell compartment. Subsequently, suicide of the committed compartment occurred and death of the dogs ensued.

These findings confirmed earlier studies by Jemionek et al. (1982b) on CCE fractionation of dog bone marrow. They observed the complete hematopoietic reconstitution of dogs, lethally irradiated with 9.0 Gy gamma and transfused with cells isolated in fraction I's. Lethally irradiated dogs that

received fraction III containing 60-75% of all CFU-GM activity had a mean survival time of 22 days.

As discussed earlier, the extent of physical separation of committed stems contained within fraction III and the pluripotent stem cells contained within fraction I was greater in peripheral blood than in bone marrow aspirates. The hypothetical existence of the bone marrow-blood barrier may be responsible for the homogeneous committed stem cell populations effecting the magnitude of separation of these populations in peripheral blood. One is drawn to speculate at this point, on the possibility of whether the magnitude of the physical separation is reinforced by a homogeneous population of circulating pluripotent stem cells.

Inoue et al. (1981) have reported the separation and concentration of the murine pluripotent hematopoietic stem cell (CFU-S) using a combination of density gradient centrifugation sedimentation (DGCS) and counterflow centrifugation elutriation. After CCE fractionation of the DGCS fractions with the highest CFU-S activity, it was determined that the highest CFU-S activity was associated with nucleated cells with cell volumes ranging between 100 and 150 μm^3 . Assuming a species variation in CFU-S volume between murine, canine, and human, the data of the mean volume of fraction I in dogs (131-135 μm^3) and humans (173 μm^3) are in agreement with Inoue et al.

The incomplete separation of the committed stem cells from the pluripotent stem cells was observed on one occasion, as characterized by an unusually high lymphocytic percentage in fraction III and by committed stem cell culture assays denoting that little separation had occurred. Transplantation of this fraction resulted in a hematopoietic recovery profile indicating the presence of the pluripotent stem cell in fraction III (Figure 32). This particular study provided further evidence of the validity of the physical separation of the peripheral blood hematopoietic stem cells and the reliability of the assays used to

monitor this separation.

The physical separation of the unipotent and pluripotent stem cells by elutriation is a further confirmation of the many observations made by other investigators. Data obtained from spleen colony assays suggest that circulating CFU-GM are members of a relatively late generation (Gidali et al., 1974), with a lower capacity for self-renewal than their bone marrow counterparts (Micklem et al. 1975). Hematopoietic studies by Worton et al. (1969), Sutherland et al. (1971) and Jacobsen et al. (1979) on bone marrow aspirates have shown, that for both CFU-S and CFU-GM, subpopulations of relatively small cell size have a higher capacity for self-replication than those of larger cells. Simply stated, the younger CFU-S with self-renewal capabilities was found to be smaller than the more mature CFU-GM.

Previous attempts to separate committed stem cells physically from pluripotent stem cells, by other separation techniques, also support our findings. Most of these separation attempts utilized, singly or in tandem, two separation techniques. The first being density gradient centrifugation sedimentation, a method in which cells are spun to their equilibrium position in a gradient of bovine serum albumin (Leif and Vinograd, 1964; Turner et al., 1967). The second technique was velocity sedimentation, a method that uses differences in sedimentation rates to separate cell on the basis of size (Miller and Phillips, 1969).

Using these techniques Worton et al. (1969) separated mouse bone marrow CFU-S from CFU-GM. They showed a distinct difference in their densities, with the more mature CFU-GM having a higher density than the younger CFU-S. Furthermore, the velocity sedimentation studies showed that the committed CFU-GM population had a higher sedimentation velocity than the CFU-S, and on these basis the CFU-GM was again shown to be larger than the CFU-S. Haskill et al. (1970), using density distribution analysis, have shown that

in resting murine bone marrow, populations of high density in vitro CFC's (CFU-GM) were devoid of significant in vivo activity (CFU-S). However, upon bone marrow stimulation with Freund's adjuvant medium, the in vivo CFC (pluripotent stem cells) increased significantly in density, while the in vitro CFC (committed stem cells) did not significantly change in density but increased significantly in total number. These data indicated that because the in vivo CFC were in G_0 resting phase or in long cycles very few pluripotent stem cells were in the higher density phase, while the more active cycling committed stem cells were in the higher density phase.

Visser et al. (1980) using a light activated cell sorter have reported cell diameters of various murine hematopoietic progenitor cells. They report the diameter of the CFU-GM to be greater than that of the CFU-S.

Of the various endeavors to separate physically hematopoietic progenitor cells, whether its pluripotent from committed stem cells as mentioned above, or separation of subpopulations of CFU-S (Monette et al., 1974; Visser et al., 1977) or even subpopulations of CFU-GM (Bols et al., 1979; Williams and van der Engh, 1975; Williams and Moore, 1973), they all have one thing in common. They were utilizing two basic physical properties of the cells in question, density and size, which is comprised of shape and volume, to achieve the separations. The principle of elutriation magnifies the subtle physical property differences (density, shape, and volume) to effect a more significant separation. In effect, elutriation is conducting the functions of density gradient sedimentation and velocity sedimentation, simultaneously. This capability of the elutriation technique has in essence allowed us to separate fully the pluripotent stem cell, not only from the CFU-GM, but from the M-CFC, CFU-F and the CFU-M committed stem cells as well.

The physical separation of committed stem cells but not pluripotent stem cells from the immunocompetent lymphocytes by counterflow centrifugation elutriation did not afford us the capability to perform allogeneic transplantation studies as had been hoped earlier. Other separation techniques, including the re-elutriation of fraction I have been proposed for the possible separation of the pluripotent stem cells from the immunocompetent cells in fraction I.

Effects of Irradiation Dose and Support on Dog Survival

Canine studies (Furth et al., 1952; Coulter et al., 1952; Sorensen et al., 1960) indicated that antibiotics, singly or in combination, appear to be somewhat effective in the LD₅₀ range; however, antibiotics alone or in combination with stored whole blood was ineffective in reducing the mortality rate when the irradiation dose approached the LD₁₀₀ range. The studies of Cronkite et al. (1954), Wood et al. (1953), Jackson et al. (1959), and Fliedner et al., (1958) have showed that the principle defect responsible for postirradiation hemorrhage may be thrombocytopenia and have shown that transfusions of fresh platelets will control postirradiation hemorrhage. Conrad et al. (1956) showed the beneficial effects of supportive fluid therapy can extend the life of the irradiated dog beyond the time period associated with mortality from the gastrointestinal syndrome.

Sorensen et al. (1960) utilizing an ambitious support regimen which included all the above protocols were able to reduce substantially the mortality rate of dogs exposed to 400 rad (4.0 Gy) of x-irradiation measured free-in-air at the position corresponding to the proximal skin surface. The support regimen employed for our studies was similar to that of Sorensen et al. (1960). However, at 6.0 Gy mid-line tissue dose the support regimen did not effect the mortality rate of our dogs.

The irradiation dose received by our experimental beagles was derived from the following criterion. The dose had to be significantly above the LD_{100} to insure killing of the hematopoietic stem cells, and low enough to avoid gastrointestinal complications and hematopoietic microenvironmental injury. George et al. (1968) and Pitchford and Thorp (1968) exposed beagles to mixed gamma-neutron irradiation and/or 250 KVP x-irradiation at the Armed Forces Radiobiology Research Institute and established an LD_{100} dose of approximately 285 rad (2.85 Gy) mid-line tissue dose. Jemionek et al. (1982b) exposed beagles to 9.0 Gy and observed clinical and pathological symptoms of gastrointestinal syndrome. Thus we decided on 6.0 Gy exposures. The exposure to 6.0 Gy did not manifest clinical (other than vomiting and drooling) or pathological evidence of gastrointestinal involvement in necropsied dogs.

A significant advantage of reducing the irradiation exposure to 6.0 Gy is evident from Table 12. Exposure to 9.0 Gy required significantly higher CFU-GM activity for survival, suggesting that significantly higher pluripotent stem cell activity was necessary for complete hematopoietic reconstitution at the higher dose exposure. These findings indicate that there was greater damage to the hematopoietic microenvironment, resulting in fewer bone marrow sites capable of supporting hematopoiesis and therefore, requiring greater numbers of circulating stem cells to seed these few active sites. These observations may also suggest greater irradiation induced injury to the animal as a whole, resulting in death of the animal despite of the bone marrow engraftment, or that at 6.0 Gy not all stem cells are destroyed, such that by themselves those stem cells remaining cannot repopulate the bone marrow but fewer transfused stem cells may be required to achieve complete engraftment.

The important new contributions of this research were: (1) the elucidation of the presence of circulating homogeneous populations of M-CFC,

CFU-F, CFU-M, as well as CFU-GM, providing further evidence for the existence of a bone marrow-blood barrier in dogs and new evidence for the existence of the same in humans, (2) the physical separation of committed stem cells from the immunocompetent lymphocytes and pluripotent stem cells, indicating that although the pluripotent and committed stem cells are morphologically indistinguishable, their physical characteristics, as ascertained by CCE, are substantially different and further suggests that the differentiation of a pluripotent stem cell into a committed stem cell is a much more altering event than the subtle change previously accepted.

SUMMARY AND CONCLUSION

The counterflow centrifugation elutriation (CCE) fractionation of human spontaneously circulating stem cells revealed the presence of homogeneous subpopulation of granulocyte/macrophage (CFU-GM) and macrophage (M-CFC) progenitor cells within the peripheral blood. This was an original observation of the present study. These circulating committed stem cells are subpopulations of the more heterogeneous populations residing within the bone marrow. These data are highly indicative of the existence of a bone marrow-blood barrier in humans capable of selecting specific stem cell populations for mobilization into peripheral blood.

Elutriation fractionation studies of unmobilized and dextran sulfate mobilized peripheral blood stem cells of dogs brought to light the presence of homogeneous populations of CFU-GM, M-CFC, CFU-F (stromal cell precursors) and CFU-M (megakaryocytic precursors). These findings confirm the observations of others who reported the homogeneity of CFU-GM populations in peripheral blood of dogs and support the existence of the postulated bone marrow-blood barrier able to select a specific CFU-GM subpopulation from the bone marrow for migration into the peripheral blood. Our new observations, however, denote that the hypothesized bone marrow-blood barrier, in dogs, was selective not only for CFU-GM, but also for M-CFC, CFU-F, and CFU-M. Analogous data derived from CCE fractionation of CFU-GM and M-CFC obtained from peripheral blood of normal and dextran sulfate recipient dogs, support the contention that the bone marrow-blood barrier is unaltered by the stem-cell-migration-enhancement effect of dextran sulfate.

Fraction I autologous transfusions, containing less than 4% of all assayed committed stem cell activity, were capable of repopulating both the myeloid and

lymphoid cell lines in the bone marrow of lethally irradiated dogs. These data are indicative of the presence of the primitive pluripotent stem cells. Fraction III autologous transfusions, containing greater than 95% of all measured committed stem cell activity, were not able to achieve the complete hematopoietic reconstitution of recipient lethally irradiated dogs. The fractional transfusions studies are indicative of the clean separation of committed stem cells from the pluripotent stem cells. These data indicate that although the pluripotent and committed stem cells are morphologically indistinguishable, their physical characteristics are sufficiently different to suggest that the differentiation of a pluripotent stem cell into a committed stem cell is a more altering event than previously acknowledged. Furthermore, the committed stem cells were isolated in high concentrations with low residual numbers of immune competent cells.

Dogs exposed to 9.0 Gy required higher numbers of pluripotent stem cells for successful bone marrow engraftments. These findings denote a greater hematopoietic environment injury resulting in fewer bone marrow sites able to support hematopoiesis. Thus, requiring higher numbers of circulating stem cells to seed those few active sites. Other possible explanations are that the greater irradiation induced damage to other vital tissues producing death despite a successful engraftment or that 6.0 Gy exposures result in incomplete killing of hematopoietic stem cells, which are too few to replenish the bone marrow alone, but require fewer transfused stem cells to achieve complete engraftment.

In conclusion, our findings present evidence for the existence of a bone marrow-blood barrier in both human and canine that permits the passage of homogeneous subpopulations of committed unipotent stem cells from the bone marrow into the peripheral circulation. The definitive separation of larger numbers of committed stem cells from pluripotent stem cells opens a multitude of investigational possibilities. One that comes to mind is the determination of

specific stimulating factors that will induce committed stem cell proliferation or differentiation but not that of pluripotent stem cells, or vice versa. Further cellular characterizations of respective stem cell populations are now also possible.

Appendix 1. Consent for performance of apheresis procedure for the
Experimental Hematology Section, Pediatrics Oncology
Branch, National Cancer Institute, National Institute of
Health, Bethesda, Maryland

CONSENT FOR PERFORMANCE OF APHERESIS PROCEDURE

Procedure (check appropriate box)

<input checked="" type="checkbox"/> Leukocytapheresis	<input type="checkbox"/> Thrombocytapheresis
<input type="checkbox"/> Lymphocytapheresis	<input type="checkbox"/> Neocytapheresis
<input type="checkbox"/> Granulocytapheresis	<input type="checkbox"/> Monocytapheresis
<input type="checkbox"/> Plasmapheresis	<input type="checkbox"/> Other _____

This procedure will be done using standard methods. A needle(s) will be inserted in a vein in your arm(s) and blood will be withdrawn. An agent (which may vary with the procedure) will be added to prevent clotting. Your blood will be separated by spinning it to collect

(Blood component(s) to be retained)

and the remainder returned to you through ☐ the same needle ☐ the needle in your other arm.

You have been selected as a donor because:

Your _____ are needed ☐ for transfusion to a patient who requires this specific component.
☐ for research into human health and disease.

This procedure has been performed many times without incident, but occasionally minor side effects may be experienced such as nausea, vomiting, fainting, chills, fever, bruising at the needle site or loss of one pint of your blood. Rarely, there are more serious side effects such as seizures, infection, the introduction of air into your blood stream or bleeding from anticoagulation. If this is a manual apheresis procedure, the possibility exists that an error could be made in the re-infusion of blood or blood components, leading to the transfusion of someone else's blood. To minimize this risk, you will be asked to identify your signature on each container before re-infusion is begun. Should any of these unexpected reactions occur, appropriate medical measures will be initiated.

Check if appropriate:

☒ Granulocytapheresis only: For this procedure it may be necessary to infuse a red blood cell sedimenting agent - 6% Hydroxyethyl starch. A small number of allergic reactions have been reported to this agent.

This procedure will take approximately _____ hours, and will be done _____ time(s) in the Clinical Center Blood Bank Apheresis Unit.

Any records kept by NIH in connection with this procedure will be maintained in accordance with the Privacy Act.

No benefits will accrue to you or to your family from your participation in this procedure, except the knowledge that you have contributed to the advancement of health care at NIH.

Short term medical care will be provided for medical complications resulting from this procedure, but neither the Clinical Center, nor the Federal Government, will provide financial compensation or long term medical treatment for such injuries except as may be provided through whatever remedies are normally available under law. You, or your representative, may withdraw consent or refuse participation in this procedure(s) for any reason, and at any time, either verbally or in writing, without prejudice or penalty. Should you decide to withdraw consent during this procedure, it can safely be interrupted and your blood re-infused.

A physician is available in the Blood Bank if you have any questions, or if you would like more information about this procedure.

If you experience any unusual side effects as a result of this procedure after leaving this unit, you may contact Dr. Harvey Klein or another Blood Bank physician at 496-4506. After 9:00 p.m. and on weekends, call the NIH operator at 496-1211 for the name and phone number of the Blood Bank physician "on-call".

I, the undersigned, understand:

1. the nature of the procedure, as explained to me.
2. that I may withdraw my consent at any time, for any reason, without prejudice, either verbally or in writing.
3. the potential and inherent risks of this procedure and the medical assistance which is available to me.
4. that my decision to participate in this procedure, as described above, is confirmed by my signature.

Signature of Witness

Date

Signature of donor

Date

Signature of Person Performing Procedure

Date

Appendix 2. Donor medical history and statement of consent record for leukapheresis volunteers at the Experimental Hematology Section, Pediatric Oncology Branch, National Cancer Institute, National Institute of Health, Bethesda, Maryland

NIH-2200-5
8-77

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL INSTITUTES OF HEALTH

DONOR MEDICAL HISTORY AND STATEMENT OF CONSENT RECORD

(For Leukapheresis by Continuous Flow Centrifugation, or by Leukopak Filters)

HISTORY	YES	NO	HISTORY	YES	NO
1. Blood Donor Rejection (Specify reason):			13. Ulcers of the Stomach or Intestine		
			14. Blood Disease		
2. Hepatitis (Exposure within 6 months)			15. Bleeding Problems		
3. Yellow Jaundice or Liver Problems			16. Convulsions or Fainting Spells		
4. Venereal Disease			17. Blood or Plasma Transfusion		
5. Tuberculosis			When:		
6. Heart Disease			How Many:		
7. Rheumatic Fever			18. Medications Taken Regularly (Specify Kind):		
8. Kidney Disease			19. Allergies (Specify Type):		
9. Shortness of Breath			a. Fish		
10. High Blood Pressure			b. Penicillin		
11. Diabetes			c.		
12. Malaria			d.		

I will notify plasmapheresis personnel if any of the following conditions exist at the time of a scheduled donation.

1. Head or Chest Cold	6. Recent Operation Within 6 Months (Specify Type):
2. Gastro-intestinal Upset	
3. Skin Infections	7. Tooth Extraction or Dental Prophylaxis
4. Antibiotic Treatment Within 2 Weeks	8. Recent Immunizations (Specify Type):
5. Pregnancy Within 6 Months	

CERTIFICATION

I hereby voluntarily give permission to the Medical Officers and employees of the National Institutes of Health to withdraw my blood for separation by continuous flow centrifugation or by Leukopak filters and transfusion of my red blood cells and plasma back to me with removal of buffy coat cells.

I certify that I have truthfully and fully answered all questions addressed to me regarding my present and prior illness, symptoms, or physical condition. I understand the procedure fully and realize this is a research procedure and I will not necessarily derive benefit from it.

SIGNATURE OF DONOR	DATE
SIGNATURE OF WITNESS	DATE

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